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THE SEROLOGICAL DIFFERENTIATION OF SMOOTH STRAINS OF THE BRUCELLA GROUP.

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IT is a curious anomaly that, while serological methods have proved of such value in the classification of many groups of organisms, they should so far have failed with the *Brucella* group. Working with the agglutination and the absorption of agglutinins test, different workers have obtained discordant results. Feusier and Meyer (1920) divided the members of the *Brucella* group into four types, Khaled (1921) into three types, Evans (1925) into eight types, Burnet (1925) into two types, and Ross (1927) into four types. Neither Orcutt, (1926), Weigmann (1931) nor Kristensen (1931) could find any serological distinction between *abortus* and *melitensis* strains, while Bieling (1930) and Olitzki and Bromberg (1931) claimed to be able to differentiate these organisms by absorption tests.

A strong faith in the value of antigenic analysis prompted us to seek for the cause of these discrepancies. The work that is now described affords, we believe, a solution to this hitherto perplexing problem.

DESCRIPTION OF STRAINS USED.

Fifty-one strains of bovine *abortus* type, of which twenty-two were isolated from cattle or cows' milk in England, Rhodesia, Palestine and the U.S.A., and thirteen from the blood of human patients with undulant fever in England, Denmark, Rhodesia and the U.S.A.; sixteen were of uncertain origin.

Twelve strains of porcine *abortus* type, of which nine were from pigs in Denmark and the U.S.A., and one from man in the U.S.A.; two were of uncertain origin.

Forty-four strains of *melitensis* type, of which two came from goats in Malta and Tunis, thirty-one from man in Tunis, Malta, Palestine, India, Rhodesia, Southern France, and Peru; eleven were of uncertain origin.

Eleven *paramelitensis* strains, of which seven were from goats in Tunis, and one from man in Malta; three were of uncertain origin.

TECHNIQUE AND RESULTS OF PRELIMINARY EXPERIMENTS.

Agglutinating sera were obtained against seventeen of these strains by the injection of rabbits. Both for inoculation, for agglutination, and for absorption, antigens were prepared by growing the organisms on heart extract agar for two to three days at 37° C., washing off with 0·25 per cent. formolized saline, heating to 60° C. for one hour, and diluting to the required strength with 0·25 per cent. formolized saline. Agglutinating suspensions were standardized by opacity to a strength corresponding to 1000 million *Bact. coli* per c.c.; absorbing suspensions were standardized to 150,000 million per c.c. All agglutination tests were carried out by preparing dilutions of serum in geometrical series, mixing with an equal quantity of antigen in Dreyer's tubes, and incubating in a water-bath at 55° C. for eighteen hours. The tubes were examined with a low-power magnifying-glass against an illuminated dark background, and the results were recorded by means of a notation corresponding in essence to that originally described by Dreyer and Inman (1917) for organisms of the typhoid-paratyphoid group. In order to make the readings as exact as possible, numerous preliminary experiments were carried out with different sera, using arithmetical dilutions, and different antigens, to obtain the necessary interpolation factors. The technique of the absorption test will not be described in detail, since it was subsequently modified; suffice it to say that the serum was absorbed in a dilution of 1/256 of the titre with an equal volume of suspension containing 150,000 million organisms per c.c., added fractionally in three separate portions at two-hourly intervals; that the tubes were incubated for six hours at 37° C. and for eighteen hours in the ice-chest; and that the supernatant fluid, together with an unabsorbed control, was titrated out against the homologous and the heterologous strains.

The seventeen sera were absorbed, each with about sixty different strains, and the proportion of agglutinins removed was tabulated in percentages. The results were confusing. While with some sera it seemed possible to draw a fairly clear distinction between *abortus* and *melitensis* strains, with others this proved impossible. Our findings were in fact similar to those reported by several previous workers, and led us to doubt whether serological methods were likely to prove of value in the classification of the *Brucella* group.

The key to the problem was afforded by an investigation into non-specific agglutination carried out more or less simultaneously by Pandit and Wilson (1932). It was found that many of the strains with which we were working, particularly of the *melitensis* type, were rough; that is to say, they were thermo-agglutinable, they were sometimes agglutinated by salt, and they were often agglutinated markedly by acid. It was further noticed that these rough strains, no matter what their origin might be, were agglutinated by a *paramelitensis* serum, but not by a serum prepared against a recently isolated *abortus* strain, while strains which were partly rough were agglutinated by both sera. This indicated that rough strains probably shared a common antigen. If this was true, it would follow that a serum prepared against a partly rough strain would contain both smooth and rough agglutinins; and since the rough antigen appeared to be common to all types, no sharp differentiation would be obtained by the use of such a serum.

In order to ascertain whether specific differences did exist between the different types, it was evident that for the preparation of agglutinating sera only strains must be used which were absolutely smooth.

Examination of the results obtained in the absorption experiments that have already been described showed that all of the five *melitensis* sera employed had been prepared by the injection of strains which were partly or completely rough; that one out of the nine bovine *abortus* strains used for serum preparation was rough, and that one out of the two porcine *abortus* strains was rough. It was subsequently found that one of the bovine *abortus* strains used for serum preparation was a mixed strain containing both bovine *abortus* and *melitensis* organisms.

TECHNIQUE OF MAIN EXPERIMENTS.

Fresh sera were prepared against a number of recently isolated smooth strains of *melitensis* and of porcine *abortus*, and seventeen smooth strains representative of the different types were selected for intensive study by the absorption test. The origin of these seventeen strains was as follows:

1. K 25 .	Bovine *abortus*	of	human	origin	from Denmark.
2. Little .	,,	,,	,,	,,	England.
3. M 736 .	,,	,,	bovine	,,	,,
4. America 1	,,	,,	,,	,,	U.S.A.
5. America 3	,,	,,	human	,,	,,
6. Enslin .	,,	,,	,,	,,	Rhodesia.
7. P 1 .	Porcine	,,	porcine	,,	New York State, U.S.A.
8. P 404 .	,,	,,	,,	,,	Missouri, U.S.A.
9. P 16 .	,,	,,	,,	,,	Denmark.
10. P 21 .	,,	,,	,,	,,	,,
11. Lisbonne 3	*Melitensis*	,,	human	,,	France.
12. Lisbonne 5	,,	,,	,,	,,	,,
13. Y 6 .	,,	,,	,,	,,	Palestine.
14. Y 12 .	,,	,,	,,	,,	,,
15. Page .	,,	,,	,,	,,	Malta.
16. Loiseau	,,	,,	,,	,,	Peru.
17. Caprine 2222	,,	,,	caprine	,,	Malta.

In order to economize time and material the technique of the absorption test was modified; after a number of preliminary trials the following procedure was found most satisfactory, and was adopted for all subsequent experiments. The serum was diluted to 1/64 of the titre (*i. e.* a serum with a titre of 1/640 was absorbed at 1/10), and was mixed with an equal volume of absorbing suspension standardized to an opacity corresponding to 3000 million *coli* per c.c. The mixture was incubated for 2 hours at 37° C., being shaken gently from time to time, centrifuged at 4000 r.p.m. for 45 minutes, and the supernatant fluid, together with the unabsorbed control serum, was titrated against the homologous and heterologous strains. Agglutination tests were

TABLE 1.—*Absorption Tests with a Bovine* Abortus *Serum* (*K25*).

Antigen.	Unabsorbed.	Absorbed at 1/40 with—																
		K25.	Little.	M736.	America 1.	America 3.	Enslin.	P1.	P404.	P16.	P21.	Lisbonne 3.	Lisbonne 5.	Y6.	Y12.	Page.	Loiseau.	Caprine 2222.
Abortus bovine K25 .	S2560	0	0	0	0	0	0	0	S320	0	0	0	0	S1280	S2560	S2560	S2560	S2560
,, ,, Little .	S2560	..	0															
,, ,, M736 .	T−1280 tr 2560	..	..	0														
,, ,, America 1	T−1280	..	..	..	0													
,, ,, ,, 3 .	T−1280 tr 2560	..	..	..	..	0												
,, ,, Enslin .	T−2560	..	..	..	..	..	0											
,, porcine P1 .	T−2560	..	..	..	..	..	..	0										
,, ,, P404 .	S+2560	..	..	..	..	..	..	..	S320									
,, ,, P16 .	T−2560	..	..	..	..	..	..	..	..	0								
,, ,, P21 .	S+2560	..	..	..	..	..	..	..	..	..	0							
Melitensis Lisbonne 3 .	S2560	..	..	..	..	..	..	..	..	..	..	0						
,, ,, 5 .	S+1280 tr 2560	..	..	..	..	..	..	..	..	..	..	..	0					
,, Y6 . .	S640	..	..	..	..	..	..	..	..	..	..	..	..	0				
,, Y12 . .	S640	..	..	..	..	..	..	..	..	..	..	..	..	..	0			
,, Page . .	T−320	..	..	..	..	..	..	..	..	..	..	..	..	..	..	0		
,, Loiseau .	S+320 tr 640	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	0	
,, Caprine 2222 .	S+640	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	0

T− = Almost complete sedimentation with agglutination in the form of large, irregularly-sized granules or floccules; marked clearing of the supernatant fluid.

S+ = Partial sedimentation with agglutination in the form of fairly coarse, often irregularly-sized granules; partial clearing of supernatant fluid.

S = Slight sedimentation with agglutination in the form of fine granules of uniform size, just visible to the naked eye; very little clearing of the supernatant fluid.

tr = Usually no sedimentation, with agglutination in the form of very fine granules visible with a magnifying glass; no appreciable clearing of the supernatant fluid.

TABLE II.—*Absorption Tests with a* Melitensis *Serum* (*Y6*).

Antigen	Unabsorbed.	Absorbed at 1/10 with—																
		Y6.	K25.	Little.	M736.	America 1.	America 3.	Enslin.	P1.	P404.	P16.	P21.	Lisbonne 3.	Lisbonne 5.	Y12.	Page.	Loiseau.	Caprine 2222.
Melitensis Y6 . . .	S640	0	S160	S160	S160	S160	S160	S160	S+80	S160	S+160	S+160	S80	tr 40	S+80	0	0	0
Abortus bovine K25 .	S+160	..	0															
,, ,, Little .	S320	..	..	0														
,, ,, M736 .	S320	..	..	..	0													
,, ,, America 1	S320	..	..	..	..	0												
,, ,, ,, 3	S320	..	..	..	..	..	0											
,, ,, Enslin	T−320	..	..	..	..	..	..	0										
,, porcine P1 .	S+320	..	..	..	..	..	..	..	0									
,, ,, P404 .	S+320	..	..	..	..	..	..	..	..	0								
,, ,, P16 .	S+320	..	..	..	..	..	..	..	..	..	0							
,, ,, P21 .	S+320	..	..	..	..	..	..	..	..	..	..	0						
Melitensis Lisbonne 3 .	S+320	..	..	..	..	..	..	..	..	..	..	..	0					
,, ,, 5 .	S+320	..	..	..	..	..	..	..	..	..	..	..	..	0				
,, Y12 . .	S640	..	..	..	..	..	..	..	..	..	..	..	..	..	T−80			
,, Page .	S640	..	..	..	..	..	..	..	..	..	..	..	..	..	..	0		
,, Loiseau . .	S640	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	0	
,, Caprine 2222	T−640 tr 1280	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	0

TABLE III.—*Synoptic Table Recording Percentage of Agglutinins Removed by Absorption of Thirteen Sera with Seventeen Different Strains.*

Antigens.	Sera.												
	K25.	Little.	M736.	America 1.	America 3.	Enslin.	P1.	P404.	P16.	Lisbonne 1.	Lisbonne 3.	Y6.	Y12.
Abortus bovine K25 . .	100	100	100	100	100	100	100	100	100	100	100	75	88
,, ,, Little . .	100	100	100	100	100	100	100	100	100	100	100	75	75
,, ,, M736 . .	100	100	100	100	100	100	100	100	100	100	100	75	75
,, ,, America 1 .	100	100	100	100	100	100	100	100	100	100	100	75	75
,, ,, ,, 3 .	100	100	100	100	100	100	100	100	100	100	100	75	75
,, ,, Enslin . .	100	100	100	100	100	100	100	100	100	100	100	75	82
,, porcine P1 . . .	100	100	100	100	100	100	100	100	100	100	100	83	88
,, ,, P404 . .	100	100	100	100	100	100	100	100	100	100	100	75	77
,, ,, P16 . .	100	100	100	100	100	100	100	100	100	100	100	65	29
,, ,, P21 . .	100	100	100	100	100	100	100	100	100	100	100	65	75
Melitensis Lisbonne 3 . .	100	97	100	100	100	100	100	100	100	100	100	89	96
,, ,, 5 . .	100	100	100	100	100	100	100	100	100	100	100	92	96
,, Y6 . . .	50	64	75	35	53	57	75	73	85	50	91	100	100
,, Y12 . . .	29	7	75	50	42	53	75	47	57	75	84	100	100
,, Page . . .	50	63	75	35	61	53	81	75	75	54	93	100	100
,, Loiseau . . .	50	30	83	30	53	53	88	64	75	50	88	100	100
,, Caprine 2222 . .	29	50	75	7	46	50	71	35	65	54	84	100	100

carried out, as before, in Dreyer's tubes, and incubation was for 18 hours at 55° C.

Altogether thirteen sera were absorbed with seventeen separate strains. Space prevents the reproduction of the protocols. Two tables recording the actual readings will be given, representative of tests carried out with a bovine *abortus* and a *melitensis* serum, and a third synoptic table comprising the percentage of agglutinins absorbed in the whole series.

Table I shows that a bovine *abortus* serum agglutinates bovine and porcine *abortus* strains up to half or full titre, while *melitensis* strains react to only an eighth or a quarter titre. Bovine and porcine strains remove all the agglutinins from the serum, whereas *melitensis* strains remove at the most only 50 per cent. In this respect the two Lisbonne strains behave like the *abortus* type.

The results with a porcine *abortus* serum were very similar to those depicted in Table I; thus bovine and porcine *abortus* strains were agglutinated to half or full titre by a porcine *abortus* serum, while *melitensis* strains were agglutinated to only a quarter titre. All agglutinins were removed by absorption with bovine and porcine strains, but not more than 50 to 88 per cent. by *melitensis* strains. Again the two Lisbonne strains behaved like the *abortus* type.

Table II shows that bovine and porcine *abortus* strains react to about half titre with a *melitensis* serum, while *melitensis* strains react to full titre. While all agglutinins are removed by absorption with *melitensis* strains, only 75 to 84 per cent. are removed by bovine and porcine *abortus* strains. The Lisbonne strains seem to be intermediate between *abortus* and *melitensis* strains.

Table III needs rather more discussion. It will be seen that all bovine and porcine *abortus* strains remove all agglutinins from bovine and porcine *abortus* sera. The Lisbonne strains and sera react in the same manner. (It may be mentioned that Lisbonne 1 strain was not included in the absorbing strains because of its low absorptive capacity, even for its own serum. In its differential absorption, however, of *abortus* and *melitensis* sera it behaved essentially like an *abortus* strain.) Bovine *abortus* strains remove about 75 per cent. of agglutinins from *melitensis* sera, the American porcine *abortus* strains (P 1 and P 404) about 80 per cent., and the Danish porcine *abortus* strains (P 16 and P 21) about 65 per cent.

All *melitensis* strains absorb all the agglutinins from both the *melitensis* sera tested; they absorb, however, only about 50 per cent. of agglutinins from the bovine *abortus* and about 75 per cent. from the porcine *abortus* sera. From this table it is clear that the strains fall antigenically into two main groups, the one group containing the bovine and porcine *abortus*, the other group the *melitensis* strains.

EXPERIMENTS MADE TO DETERMINE THE ANTIGENIC STRUCTURE OF THE *BRUCELLA* GROUP.

The work hitherto recorded in this paper renders it evident that, though *abortus* and *melitensis* strains can be differentiated serologically, there is a considerable group affinity between them. Whether the difference depends on the possession, over and above a common group antigen, of an antigen

specific for each type—as, for example, occurs in members of the Salmonella group—or whether it is determined by the existence of the same antigens in varying amount: whether, that is to say, the difference is qualitative or quantitative, it is impossible to tell. In order to gain some light on this matter cross-absorptions were carried out, using absorbing suspensions of

TABLE IV.—*Showing Complete Cross-absorption by the Use of Very Heavy Suspensions.*

Nature of experiment.		
Bovine *abortus* K25 serum absorbed with a *melitensis* strain	Titre of unabsorbed serum	S2560
	Titre after absorption in 1/40 dilution with 3000 million Y6	S1280
	Titre after absorption in 1/40 dilution with 150,000 million Y6	tr 160
	Titre after absorption in 1/40 dilution with 300,000 million Y6	0
Melitensis Y6 serum absorbed with a bovine *abortus* strain	Titre of unabsorbed serum	S640
	Titre after absorption in 1/10 dilution with 3000 million K25	S160
	Titre after absorption in 1/10 dilution with 150,000 million K25	0
	Titre after absorption in 1/10 dilution with 300,000 million K25	0
Melitensis Y6 serum absorbed with a porcine *abortus* strain	Titre of unabsorbed serum	S640
	Titre after absorption in 1/10 dilution with 3000 million P404	S160
	Titre after absorption in 1/10 dilution with 300,000 million P404	0
Melitensis Y6 serum absorbed with a *melitensis* Lisbonne strain	Titre of unabsorbed serum	S640
	Titre after absorption in 1/10 dilution with 300,000 million Lisbonne 1	0
Porcine *abortus* P404 serum absorbed with two *melitensis* strains	Titre of unabsorbed serum	S+256 tr 512
	Titre after absorption in 1/4 dilution with 3000 million Y6	T-64 tr 128
	Titre after absorption in 1/4 dilution with 3000 million Page	T-64
	Titre after absorption in 1/4 dilution with 300,000 million Y6	0
	Titre after absorption in 1/4 dilution with 300,000 million Page	0
Melitensis Lisbonne 1 serum absorbed with three *melitensis* strains	Titre of unabsorbed serum	S+320 tr 640
	Titre after absorption in 1/10 dilution with 3000 million Y6	T-160
	Titre after absorption in 1/10 dilution with 3000 million Y12	S+80 tr 160
	Titre after absorption in 1/10 dilution with 3000 million Page	S+160
	Titre after absorption in 1/10 dilution with 300,000 million Y6	0
	Titre after absorption in 1/10 dilution with 300,000 million Y12	0
	Titre after absorption in 1/10 dilution with 300,000 million Page	0

varying strengths. Assuming the correctness of our present conception of the absorption test and assuming that non-specific absorption plays no large part in the result, it was argued that, if the difference was qualitative, no amount of a heterologous organism should deprive the serum of all its agglutinins; whereas if it depended merely on an altered quantitative distribution of the same antigens, then by using sufficiently large doses it should be possible to absorb all the agglutinins from a serum by a heterologous organism. The results of these experiments, in which a single absorbing dose was allowed to interact with the serum for 2 hours at 37° C., are shown in Table IV. The results are very striking, and all point in the same direction. It will be noticed that a dose of 3000 million *melitensis* removes only 50 per cent. of agglutinins from a bovine *abortus* serum; a dose of 150,000 million, however, removes 95 per cent., and one of 300,000 million removes all the agglutinins. Conversely a dose of 3000 million bovine *abortus* removes 75 per cent. of agglutinins from a *melitensis* serum, while a dose of 150,000 million removes all the agglutinins. Similarly with a porcine *abortus* serum absorbed by a *melitensis* strain, and a *melitensis* serum absorbed by a porcine *abortus* strain, it is seen

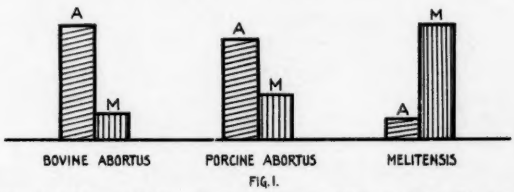

FIG. 1.

Schematic representation of antigenic structure of *Brucella* strains.

that, provided large enough doses are used, it is possible to absorb all the agglutinins from both sera with the heterologous organism. Other experiments, the details of which need not be recorded, were performed in which repeated absorption of the same serum with successive doses of the same heterologous organism was employed. The results of these experiments were in complete accord with the previous ones in which a single large dose was used.

It may therefore be concluded that the antigens present in *abortus* and *melitensis* organisms are qualitatively alike but quantitatively different.

The probable antigenic structure of these organisms is represented schematically in Fig. 1. It seems necessary to postulate the existence of only two antigens, which may be called A and M. In bovine *abortus* strains the A antigen is preponderant, the M antigen being present in comparatively small amount. In *melitensis* strains, on the other hand, the M antigen is dominant, while the A antigen constitutes but a small fraction. The amount of M antigen in bovine *abortus* strains is represented as being rather greater than the amount of A antigen in *melitensis* strains, because bovine strains are able in a given dose to absorb a higher proportion of agglutinins from a *melitensis* serum than *melitensis* strains can from a bovine serum. The porcine strains are represented as being more or less intermediate in structure between

bovine and *melitensis* strains, with, however, a greater proportion of A than of M antigen. The reason for this is that porcine strains—at least the American porcines—can absorb rather more from a *melitensis* serum than can bovine strains; and conversely *melitensis* strains can absorb rather more from a porcine serum than from a bovine serum. The difference between the porcine and the bovine strains is not very marked, but it does seem as if the porcine strains are less distantly separated from the *melitensis* strains than are the bovine strains. This conclusion derives some support from a consideration of the other attributes of these organisms. Thus the porcine resemble *melitensis* strains in their ability to grow aërobically, and in their apparently greater pathogenicity for man than bovine strains. It must be remembered, however, that there appears to be a difference between the Danish and the American porcine strains. The American porcine strains seem to be more closely allied to the *melitensis* type than do the Danish. This again may be brought into relation with the fact that while American porcine strains have given rise to numerous cases of undulant fever in man, so far no human cases have been reported in Denmark as due to infection with the porcine type. The Lisbonne *melitensis* strains occupy a peculiar position. These strains were recovered from undulant fever patients in the south of France, who were almost certainly infected from sheep or goats. So far as it is possible to judge from an epidemiological survey, they are possessed of a fairly high degree of pathogenicity for man. Antigenically they fall into the *abortus* group, but careful examination of the percentage absorption figures suggests that they must contain a considerable proportion of M antigen. It may be surmised that they are more or less intermediate between the porcine and the *melitensis* strains.

It will be realized that, apart from the two main types, represented by the bovine *abortus* strains on the one hand and the *melitensis* strains on the other, there are certain intermediate groups, containing the American and Danish porcine and the Lisbonne *melitensis* strains. This conclusion is strongly supported by a study of the H_2S formation and growth on dye plates of the various strains. The results of this study will be reported in a subsequent paper, in which it is hoped to discuss the relation between the serological and the physiological groupings. (Suffice it to say, for the moment, that both methods reveal the existence of a number of intermediate groups, generally coming from definite localities, suggesting that in a given environment races may be evolved differing in some respects from the main types.)

With regard to the antigenic structure of *para-abortus* and *paramelitensis* strains our work so far does not entitle us to speak. There seems every reason to regard these strains, as Pandit and Wilson point out, as rough variants of the parent *abortus* and *melitensis* types. The smooth antigens that are present in the parent strains are absent in their rough variants, so that no cross agglutination or cross absorption occurs between *abortus* and *melitensis* on the one hand and *para-abortus* and *paramelitensis* on the other. Since all rough variants are agglutinated by a serum prepared against *para-abortus* or *paramelitensis* strains, it is justifiable to assume that there is one antigen at least common to all these strains. Whether a second antigen exists, as in the smooth strains, and whether it is possible to differentiate between the

rough strains so as to indicate their origin, is not yet clear, but we hope that work now in hand will enable us to answer these questions.

RAPID TYPING OF BRUCELLA STRAINS BY MONOSPECIFIC SERA.

In the differentiation by the agglutinin absorption technique of organisms having one or more qualitatively different antigens in addition to the common group antigen, the dosage of absorbing organisms in relation to the titre of the serum is not of major importance; when, however, as with members of the *Brucella* group, the two main types are distinguished merely by a difference in the quantitative distribution of the same antigens, it is of supreme importance to adjust the dose of absorbing organisms to the titre of the serum in such a way as to bring out this difference most clearly. The aim must be to absorb from one serum with a heterologous organism the minor agglutinin without affecting to any great extent the major agglutinin. In our work we have obtained satisfactory results by absorbing the serum in a dilution equivalent to 1/64 titre with an equal quantity of bacterial suspension corresponding in turbidity to 3000 million *Bact. coli* per c.c. In this way we have been able to prepare monospecific sera which can be used for the rapid typing of members of the *Brucella* group. These sera were tested against all of the 118 strains in our collection. Seventeen of the strains were so rough as to have lost all their smooth antigen, and could not therefore be typed by this method. The results with the remaining strains, some of which were partly rough but still contained some of their original smooth antigen, are recorded in Table V. It will be noted that 47 out of the 48 strains of bovine

TABLE V.—*Results of Typing by Means of Monospecific Sera.*

Probable type according to origin.	Number of strains.	Serologically.	
		abortus.	*melitensis.*
Br. abortus bovine	48*	47	1
,, porcine	11	11	0
Br. melitensis	35	5	30
,, Lisbonne	6	6	0

abortus were agglutinated by a monospecific *abortus* but not by a monospecific *melitensis* serum; the single exception was a strain the exact origin of which was unknown. All of the 11 porcine *abortus* strains reacted only with an *abortus* serum. Of the thirty-five *melitensis* strains, five reacted like *abortus* strains; all of these five strains behaved like typical *abortus* strains when tested by the dye method, so that there is little doubt that their original label was incorrect. The Lisbonne *melitensis* strains reacted only with an *abortus* serum; by the dye method they resembled *melitensis* more closely than *abortus*, though showing certain points of difference from the Palestine *melitensis* type. With the exception, therefore, of the Lisbonne group of strains, which

* One strain is omitted which was agglutinated by both sera; it was plated out and, of the individual colonies that were picked off, some proved to be of the *abortus* and some of the *melitensis* type.

clearly present anomalous reactions, the use of monospecific sera rendered possible, by the simple process of direct agglutination, what appears to be a correct typing of all strains in our collection containing sufficient smooth antigen. It can therefore be recommended as a valuable method for the rapid typing of unknown strains. A warning must be issued, however, that in view of certain discrepancies between the serological and physiological methods of typing, no single method should be relied on exclusively.

SUMMARY AND CONCLUSIONS.

1. As a result of the work recorded in the present paper, and in a paper by Pandit and Wilson (1932), it is concluded that the *Brucella* group contains members which may be primarily classified into smooth and rough.

2. The smooth strains, comprising *abortus* of bovine and porcine origin and *melitensis*, are non-thermo-agglutinable, and though sometimes agglutinated slightly by acid, are not agglutinated by salt. The rough strains, comprising *para-abortus* and *paramelitensis* are thermo-agglutinable, are agglutinated strongly by acid, and not infrequently by salt.

3. In their typical forms smooth and rough strains have no serological relationship, though intermediate strains occur containing both smooth and rough antigen.

4. By the use of sera prepared against absolutely smooth strains, it is possible by the agglutinin absorption technique to divide the smooth members into two types, one type containing bovine and porcine *abortus*, the other type containing *melitensis* strains.

5. Evidence is brought to suggest that the distinction between *abortus* and *melitensis* strains is due, not to the presence of qualitatively different antigens, but to the different quantitative distribution of two common antigens.

6. Provided due regard is paid to the relationship between the absorbing dose and the titre of the serum, monospecific sera can be prepared in which the major agglutinin of the type alone persists. By means of these sera unknown strains of the *Brucella* group can be rapidly typed by direct agglutination.

7. The results of testing one hundred strains by monospecific sera are recorded, and with a single exception afforded by a group of strains from a particular locality, are seen to be in close accord with conclusions reached on epidemiological and other grounds.

8. The rough strains have not been fully studied, but it appears that there is at least one antigen common to all *para-abortus* and *paramelitensis* strains.

9. It is suggested that the reason why so many previous workers have failed to differentiate serologically between *abortus* and *melitensis* is because they have not realized the importance of using absolutely smooth strains for the preparation of their sera. Since *melitensis* strains have a marked tendency to become rough in the laboratory, it is, as a rule, difficult to obtain satisfactory sera against the *melitensis* type unless recently isolated strains are used. Unless perfectly smooth strains are employed, the resulting sera will contain some rough agglutinin, which will tend to obscure the clear differentiation of the types.

10. In so far as the smooth forms are concerned, our work confirms the conclusions previously reached by Ross (1927) as the result of examining a small number of strains.

In conclusion it is a pleasure to record our thanks to all those who have been kind enough to furnish us with *Brucella* strains, namely Dr. J. T. Duncan, Dr. G. R. Ross, Mr. L. E. W. Bevan, Dr. Martin Kristensen, Dr. Axel Thomsen, Prof. M. Lisbonne, Prof. R. Bieling, Dr. I. F. Huddleson and Dr. M. H. Soule. In particular we should like to express our extreme indebtedness to Dr. Rina Younovitch, previously of Jerusalem, for providing us with a number of freshly isolated *melitensis* strains. Had it not been for her generous co-operation, it is doubtful whether the conclusions in this paper could have been reached.

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THE " KILLING " OF BACTERIA BY BACTERIOPHAGE.

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THE lysis of bacteria by phage is so striking that it is this aspect of the action of phage on bacteria which has almost entirely been studied by workers on the subject. It is, however, possible to study the " killing " of bacteria by phage quite apart from any lytic process. This is rendered possible by the discovery of Stassano and de Beaufort (1925) that sodium citrate will inhibit the multiplication of some strains of phage; this phenomenon was further studied by Bordet and Renaux (1928), who found that oxalate had the same effect, and considered that it was due to the removal of calcium ions.

Use of Citrate.

Working with a phage active against *B. coli* in dilutions up to $1:10^{11}$, we found that in the presence of 0·75 per cent. sodium citrate the phage could neither multiply nor produce lysis ; in 0·25 per cent. citrate it could, however, do so. The coli, on the other hand, grew almost normally in 0·75 per cent. citrate broth. Agar containing 0·75 per cent. or 1 per cent. citrate was also found to give an almost normal growth of the coli, but in the presence of phage no plaques appeared at all. Experiments of Bordet's, confirmed by us, make it clear that the citrate does not inactivate the phage directly but only inhibits its multiplication.

In a culture containing coli and phage it is easy to follow the rate of multiplication of phage which can be estimated by any of the ordinary methods. Changes in the total numbers of coli present can also be followed by, for instance, a turbidity method. It has not, however, hitherto been possible to follow changes in the number of *viable* coli ; drops spread on ordinary agar plates will give no growth of organisms, as the phage can continue to develop there and to lyse the bacilli. But the use of citrate agar does afford a means of estimating the viable coli. Drops of a phage-coli mixture can be spread on citrate agar plates ; the phage will be unable to multiply further, and the colony count obtained will indicate the numbers of viable coli present.

Rapid " Killing " of Coli by Phage at Room Temperature.

The word " killing " by phage is used throughout to indicate that organisms have been so affected that they will not progressively multiply in broth nor form colonies on agar ; it is recognized that other criteria might indicate that they were not really dead. No elaborate justification of this use of the term " killing " seems to be required, for we have after all used the same criteria for determining death of organisms as have workers on the effect of antiseptics or of temperature on bacteria. We did not study the respiration of our " killed " organisms, in view of Eaton's (1931) finding that a culture lysed by phage will continue to respire actively at a time when practically all the organisms have been lysed, and are therefore certainly dead.

When a dilute suspension of coli was mixed with undiluted phage at room temperature and a drop spread on citrate agar after a few moments' contact, it was apparent next day from a colony count that over 99 per cent. of the organisms had been " killed " by this brief contact with phage. " Killing " had thus occurred under conditions where neither coli nor phage could have multiplied, nor was any method discovered by which their viability could be restored. The same result was obtained when the mixture of phage and coli was made in the presence of citrate : the citrate could thus not stop the " killing " of the coli if the phage were initially present in a sufficiently high concentration ; its action was more probably, as indicated above, to inhibit phage multiplication.

Optimum Time of Contact of Phage and Coli.

While " killing " could thus take place very rapidly, it appeared that lengthening the time of contact of phage and coli increased the number

of organisms which were " killed." This was not due to a multiplication of phage, since the same result was obtained in the cold or in the presence of citrate. Very probably an increase in the time of contact simply multiplied the chances of collision between organisms and phage " particles." Where a phage suspension containing over a million " particles " per c.c. was used, it seemed that an equilibrium was reached in about one hour at room temperature ; accordingly this period of contact was used in later experiments.

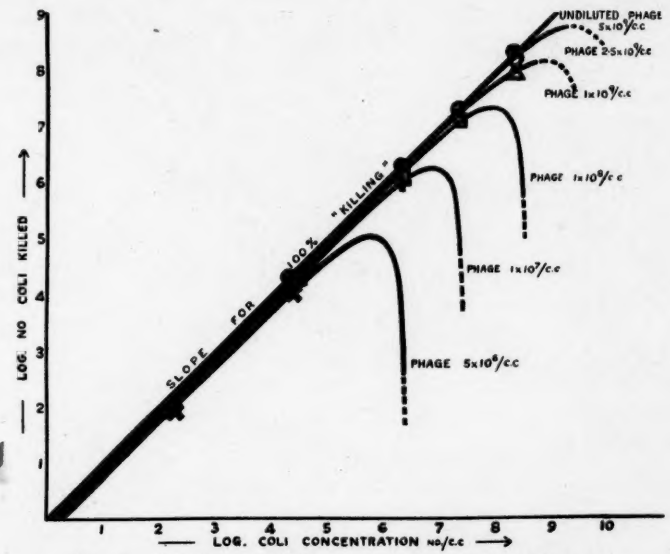

FIG. 1.—" Killing " of coli by various concentrations of phage.

We could not demonstrate that phage containing less than 10^6 " particles " per c.c. had any " killing " effect.

Amount of Phage Necessary to Bring About " Killing."

It was found that in mixtures containing less than 10^6 phage " particles " per c.c., no " killing " of coli could be detected within the limits of error of our methods. However, when the amount of phage was increased tenfold (to 10^7 " particles " per c.c.) it was able to " kill " 95 per cent. of the coli in the culture ; this was true over a range of coli concentration from 10^3 to 10^7 per c.c. ; with thicker coli suspensions it was no longer true. Coli in high concentration required comparatively more concentrated phage for comparable " killing." Our undiluted phage containing 5×10^9 " particles " per c.c. sufficed to " kill " 99 per cent. of coli in a suspension containing 5×10^8 per c.c. The absolute number of phage " particles " per c.c. seemed thus over a wide range to be more important than the ratio of phage to coli. Krueger and Northrop found that for lysis of staphylococci a definite phage : organism ratio (125 : 1) was necessary. Our failure to find that such a ratio holds in our experiments may

be due either to the fact that we were studying "killing" of coli while they studied lysis of staphylococci, or to the opportunity which our methods afforded of studying the phenomena over a much wider range of bacterial concentration than is possible by Krueger and Northrop's methods.

In Fig. 1 the numbers of coli "killed" are plotted against the initial coli concentration; each of the curves corresponds to a different dilution of a

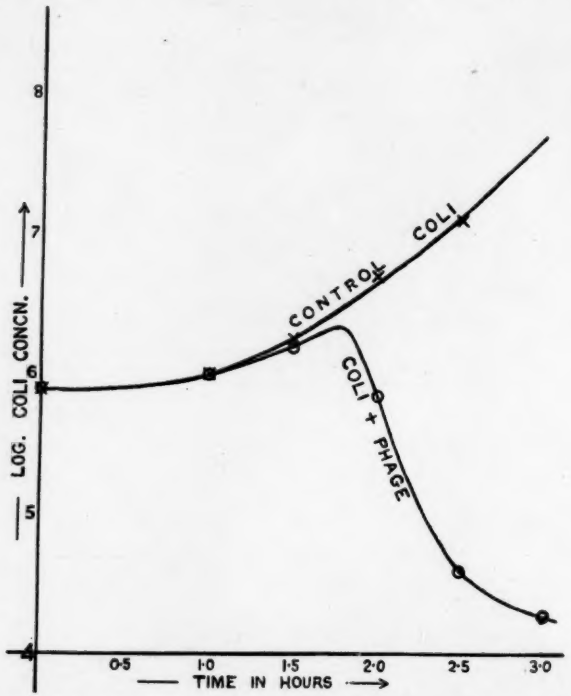

FIG. 2.—Viable coli in citrate-broth cultures with and without phage.

phage containing 5×10^9 "particles" per c.c. For each particular phage concentration there is a definite upper limit of coli concentration beyond which no appreciable "killing" can be demonstrated, but below which the percentage of organisms "killed" rises rapidly to over 95 per cent.

Fate of Coli in Cultures Containing Phage.

Cultures of coli were inoculated with dilute phage (insufficient to "kill" the organisms forthwith), and estimations of viable coli were made at intervals by plating 0·1 c.c. on to citrate agar. We found that up to a point the numbers of organisms in cultures containing phage increased at the same rate as those in a control without phage. But as a rule, before the organisms had more

than doubled their numbers the viable coli in the phage-containing tube suddenly began to fall off, and within an hour there would be hardly any left. This was true even when only a trace of phage was used to inoculate the tubes (see Fig. 2).

When the tubes contained initially about 10^8 coli per c.c. it was possible to compare the readings thus obtained with turbidity readings. It was found that after the numbers of viable coli had begun to diminish a considerable interval elapsed before lysis was perceptible—20 to 30 minutes; in fact the turbidity might be increasing at a time when the number of viable coli was diminishing. Evidently the " killed " coli did not lyse for at least 20 minutes—a fact confirmed by subsequent experiments—and the survivors continued to

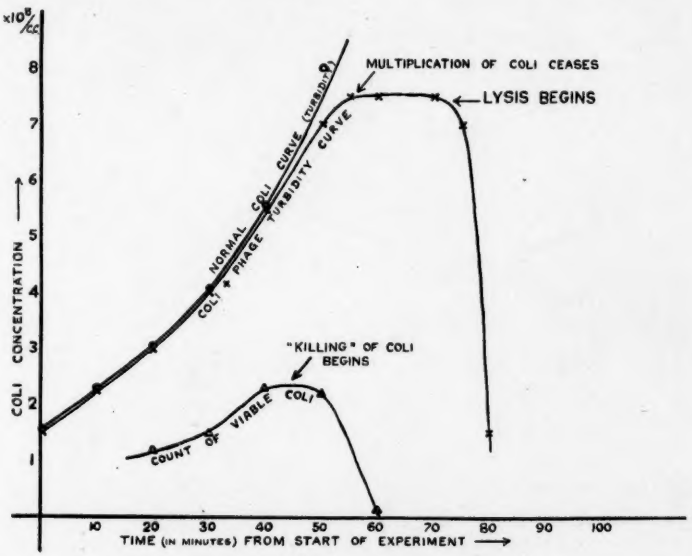

FIG. 3.—Fate of coli when incubated with phage.

increase the turbidity of the tube for a time. One experiment (see Fig. 3) showed quite clearly that at the time when the " killing " apparently began, the turbidity curve began to fall away from that of the control without phage. It is clear from the graphs that lysis, when it set in, was a much more rapid process than coli-" killing."

It seemed possible that the increase in turbidity of tubes after " killing " had begun might be due to swelling of individual coli and not entirely to increase in numbers. Accordingly observations were made in the hot-room at 37° C.; coli were watched in a counting chamber by the aid of dark-ground illumination. Counts of the numbers of coli in a given square were made every five minutes. The curve of numbers of visible organisms was found to correspond extremely closely with that of the turbidity of the tube, so that it seemed likely that the

increases in turbidity previously observed really corresponded to increases in the numbers of organisms present.

Lysis of Coli in Presence of Excess of Phage.

When undiluted phage was added to an equal volume of a coli suspension containing 10^8 organisms per c.c., it was possible to follow changes in turbidity by the aid of standard opacity tubes. Coli in the logarithmic phase of growth were used; both coli and phage were warmed to 37° before mixing and the mixture was watched in the hot-room. The increase in turbidity was frequently

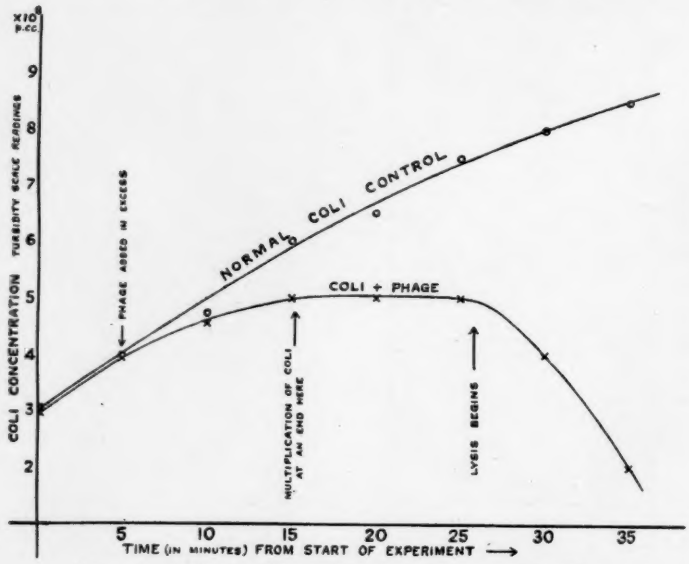

FIG. 4.—Results of adding phage in excess to a coli-culture.

observed to continue after the addition of phage; but as a rule it did not last longer than 5, or at most 10 minutes. Then, after a period during which the turbidity remained unchanged, lysis would abruptly set in after 20 minutes or a little more from the time of mixing. Earlier experiments had shown that "killing" of the coli began almost at once; so it appears, as suggested earlier, that it takes about 20 minutes for a "killed" organism to lyse. In one experiment the turbidity increase of a coli culture was watched in the hot-room; at a suitable moment one-tenth its volume of warmed phage was added to the culture in one tube and an equal volume of warmed broth to a similar control tube, and observations were continued. When the results were plotted, the curve of the phage-containing tube could be seen to part company from the control curve after 5 minutes, as Fig. 4 shows. Experiments similar to these have lately been reported by Eaton (1931).

It seems possible that the initial delay before the curves parted company might be due to the time taken for phage particles to become adsorbed to, or perhaps to enter, coli. It does not seem necessary to assume that coli rendered non-viable by phage can multiply even once before dying. This experiment, then, confirms earlier ones in showing that phage when present in excess can " kill " coli with great rapidity. It is needless to add that when phage was added to coli in such concentration that it could not immediately " kill " an appreciable number of organisms, a definite increase in turbidity occurred for some while before lysis set in.

Lysis of Organisms " Killed " at Room Temperature.

It is well known that phage will not lyse dead organisms—apart from the experiment described by Twort (1925), which is not generally admitted to

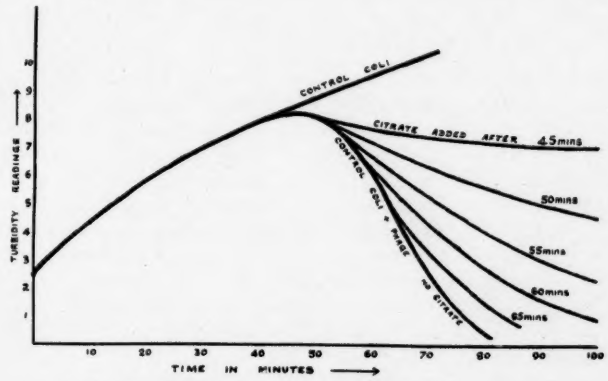

FIG. 5.—Effect of citrate on lysis of coli.

In the experiment recorded in Fig. 5 samples were removed from the coli-phage mixture every five minutes, and enough concentrated citrate was added to each to bring the citrate up to 0·75 per cent. The curves show the resulting effect on the lysis as followed by turbidity readings. When citrate was added earlier than 45 minutes no lysis occurred, and the readings followed the control coli curve.

represent true lysis by phage. It appears, however, that organisms which are " killed " as a result of the action of phage itself *are* susceptible to lysis. Coli were mixed with excess phage and held at room temperature for an hour. On transference to the 37° incubator lysis occurred, either after 20 minutes, or after a variable delay.

Lysis of Organisms at Room Temperature.

In most experiments no evidence of lysis of coli at room temperature was ever seen, even in the presence of excess of phage. Krueger and Northrop (1930) record that their staphylococcal phage in excess will produce lysis in the cold, and we can confirm this statement, for they kindly sent us their strain to study. It seems that while " killing " of coli readily and quickly occurs

at 20° C., lysis commonly does not do so. In one experiment only, lysis of coli by excess of phage occurred at 20° C. after 2½ hours.

Lysis of Organisms in the Presence of Citrate.

When excess phage was added to coli in the presence of 0·75 per cent. sodium citrate, no lysis occurred though the organisms were "killed" very quickly. Further, if a culture "killed" by phage was incubated and citrate quickly added, no lysis occurred unless the culture was just on the point of lysing at the time of adding the citrate. When this was the case the tubes became clearer, though they cleared more slowly than controls and lysis was not always complete. In the experiment recorded in Fig. 5 lysis began after 45 minutes. The turbidity curves show how the lysis was affected when citrate was added 45, 50, 55, 60 and 65 minutes respectively after the phage.

Use of Immune Serum Instead of Citrate.

Antiphage serum can be used instead of citrate to demonstrate the "killing" of coli by phage, though its mode of action is quite different. There is evidence that antiphage serum acts by uniting with phage, and so preventing it from attaching itself to or entering bacteria. Once the phage, is adsorbed to the bacteria in sufficient quantity to "kill" them, immune serum cannot save them. Its action differs from that of citrate in that when it is mixed in appropriate amount with phage and the mixture is then added to susceptible coli, these will not be "killed." In this respect it is more effective than citrate; in another respect it is less so. For if phage is added to coli in sufficient excess to "kill" them and excess of immune serum is subsequently added, incubation of the mixture will be followed by lysis after the usual 20 to 30 minutes' delay. Presumably the phage which has once entered the coli continues to multiply and lysis ensues: citrate, which perhaps penetrates within the coli where antibody cannot do so, *is* able to stop the lysis.

One uses immune serum to demonstrate the killing of coli by phage in this way: phage and coli are mixed in the appropriate amount; then, instead of plating out on to citrate agar, one part of immune serum is added to one part of the mixture, and a drop is spread on the surface of a medium in which more immune serum (33 per cent.) is incorporated with the agar. The further action of the phage is thus arrested, except perhaps as regards the lysis of those organisms which are already infected with phage, and a colony count will reveal how many coli, if any, have survived. In spite of the differences between the mechanism of action of citrate and of immune serum, colony counts so obtained corresponded very closely with those obtained by plating on to citrate agar.

The incorporation of immune serum in the medium was found to be an indispensable part of the technique. The rationale of this procedure will be discussed in a later publication; it depends upon the fact that if the agar contains no immune serum, interchange by diffusion of substances between a drop spread on the agar and fluid in the interstices of the gel is liable to upset the relations existing between the phage, the antibody and the bacteria.

Experiments with Other Phages.

A strain of phage active against *B. œrtrycke* was kindly given to us by Prof. Topley. The action of the phage was inhibited by sodium citrate, but in early experiments no evidence of immediate " killing " of *B. œrtrycke* by the phage was obtained, possibly because the phage was too weak. Later the titre of this phage was raised by passage to 2×10^9 per c.c. ; it was then found to " kill " the susceptible organism much as our coli phage did. We did not, however, make extensive quantitative studies as we had done with the coli phage.

A staphylococcal phage sent to us by Dr. A. P. Krueger was, unfortunately, not sensitive to citrate. An antiserum was prepared against it in rabbits, and with the aid of this we tried to demonstrate " killing " of staphylococci by the phage. The results were not clear-cut, and we did not succeed in convincing ourselves of the occurrence of " killing " of staphylococci apart from lysis.

Discussion.

The bacteriophage is considered by d'Herelle and many others to be a virus —an autonomous parasite. We do not consider that the results reported in this paper tell strongly either for or against this view ; but it is worth noting that the extremely rapid " killing " of *B. coli* by phage, without any accompanying multiplication of that phage, has at present no known parallel in the field of viruses attacking animals. There seems no reason to doubt that the agent which " kills " is the phage itself ; the " killing " agent was held back by a collodion membrane just tight enough to retain the phage itself, and further, it was specifically neutralized by antiphage serum.

Summary.

1. A specific phage added in excess to *B. coli* can within a few minutes, and perhaps within a few seconds, so affect the organisms that they are rendered incapable of propagation ; in other words, it apparently " kills " them immediately.

2. At 37° C. lysis of these " killed " organisms follows in 20 to 30 minutes. At 20° C., though the " killing " has taken place at once, lysis may not be demonstrable or may occur only after some hours.

3. Sodium citrate seems to act on this coli-phage by preventing its multiplication. It cannot prevent " killing " of coli if phage is added in excess, but such " killed " phage will not lyse if citrate is present.

4. The experiments described with a strain of *B. coli* and a homologous phage will not necessarily be reproducible with any other race of bacteriophage.

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PLASMA PHOSPHATASE, III: A CLINICAL METHOD FOR THE DETERMINATION OF PLASMA PHOSPHATASE.

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WE have recognized for some time that the method proposed by one of us (Kay, 1930) for the determination of the phosphatase content of blood-plasma, though sound theoretically, and capable in practice of giving dependable results, is a little tedious for routine use, and requires more time and possibly more equipment than is available in every clinical laboratory.

Since the phosphatase content of the plasma in certain cases of generalized bone disease is so much greater than that of normals and that of individuals suffering from almost all other types of disease (Kay, 1929; Roberts, 1930; Hunter, 1930), it was thought that for clinical purposes a method could be devised which, whilst possibly not so precise as that already published, might, with less expenditure of time and materials, yield results sufficiently accurate for assistance in diagnosis. The original method calls for 48 hours' hydrolysis and requires 5 c.c. of plasma, or approximately 10–11 c.c. of blood. A pH determination is also advisable. It was considered that these demands were, for clinical purposes, unnecessarily heavy.

In the method to be described, the time of hydrolysis is reduced to 3 hours, and the plasma requirement to 2 c.c. or even 1 c.c. In addition filtration of the centrifuged plasma before use is not required, nor are pH determinations, nor corrections derived from these, necessary.

In order to be able to cut down both the time of incubation and the amount of plasma, it was imperative to use a more delicate method than that of Briggs for phosphate determinations, since the amount of inorganic phosphate produced during the period of hydrolysis would necessarily be very small. After seeing the improved Kuttner-Cohen (1927) (see also Kuttner and Lichtenstein, 1930) procedure as demonstrated by Youngburg to the American Society of Biological Chemists at Montreal, April, 1931, it was decided to adapt this very delicate modification of the Denigès (1920) reaction to our requirements. To avoid pH determinations, or changes of reaction during the hydrolysis period, a strong buffer solution was used, in sufficient quantity to maintain the pH of the reaction mixture at pH 8·8, which is close to that optimal for the enzyme concerned. A glycine-NaCl-NaOH buffer, of the composition and in the proportion to be described below, was found to fulfil these requirements.

The relatively minute degree of hydrolysis obtainable with small quantities of the plasma enzyme in action for 3 hours only is to some extent offset

by working at pH 8·8, and also by using rather larger substrate concentrations. Since Mg ions under certain conditions markedly stimulate the activity of the plasma enzyme, Mg ions were added in a number of cases to the substrate (sodium β-glycero-phosphate) in optimal concentrations for this stimulus, in the hope of obtaining still greater rates of hydrolysis. The effect, under the conditions otherwise most favourable for the enzymic action, was not, however, sufficiently marked to counterbalance the added complication in what was intended to be a quick, simple method of insuring a constant [Mg″] in the reaction tubes, and the idea of adding Mg ions was therefore abandoned. For a similar reason, the idea of cutting down the incubation period to 2 hours by conducting the hydrolysis at 45° C. instead of 38° C. was not proceeded with. In most laboratories a water-bath at a temperature of 38° C. is a common article of furniture, but one at 45° C. would usually mean the devotion of a special bath to these determinations.

METHOD.

(a) *Stock Solutions Required.*

1. Sodium β-glycerophosphate (obtainable from Boots Pure Drug Co., Nottingham, England: The β–salt should be specified when ordering). 2·5 gm. of the pure crystals are dissolved in 100 c.c. of distilled water.

2. Glycine buffer solution. Analytically pure glycine (6·06 gm.) and 4·68 gm. of pure sodium chloride are dissolved in 328 c.c. of N/10 sodium hydroxide, and the solution made up to 1 litre with distilled water.

3. Sulphuric acid, 10 N. (282 c.c. of concentrated sulphuric acid made up to 1 litre with distilled water).

4. Sodium molybdate solution 7·5 per cent. As recommended by Kuttner and his co-workers (1927, 1930) Kahlbaum's "zur Analyse" or other pure product which gives no blank with the other reagents in absence of added phosphate is required.

5. Stannous chloride, 40 per cent. $SnCl_2$ in concentrated HCl solution. To be kept in the dark, in a dark-coloured bottle.

6. Standard phosphate solution, 1 c.c. contains 0·1 mgm. P. Pure dry KH_2PO_4, 0·4390 gm., is dissolved in distilled water and made up to 1 litre.

(b) *Solutions Ready For Use.*

7. Standard substrate: 1 part of 2·5 per cent. sodium β-glycerophosphate (solution 1 above) is added to 5 parts glycine buffer (solution 2 above).

8. Trichloroacetic acid, 15 per cent.

9. Molybdic acid mixture: 1 part 10N. H_2SO_4 (solution 3 above) is mixed with 2 parts distilled water and 1 part 7·5 per cent. Na_2MoO_4 (solution 4 above) is added.

10. Stannous chloride: $\frac{1}{2}$ c.c. of solution 5 above is diluted to 100 c.c. with distilled water just before using. The diluted solution can only be relied upon for *one day*.

11. Standard phosphate solutions: (*a*) 1 c.c. of standard solution 6 diluted to 50 c.c.: 5 c.c. contains 0·01 mgm. P. (*b*) 2 c.c. of solution 6 diluted to 50 c.c.: 5 c.c. contains 0·02 mgm. P. (*c*) 3 c.c. of solution 6 diluted to 50 c.c.: 5 c.c. contains 0·03 mgm. P.

Solutions 1 and 7 are kept with a little chloroform in the refrigerator. Solutions 2, 6 and 11 keep well with a little chloroform at room temperature. Solution 9 keeps fairly well for short periods. If fresh reagent is made up, the previous contents of the bottle should be thrown away and the bottle carefully rinsed out before the fresh reagent is put in.

All the foregoing materials are readily obtainable commercially.

A number of strong test-tubes (which will fit the centrifuge) or small centrifuge tubes containing 2 drops of 15 per cent. potassium oxalate solution are prepared beforehand, to hold the 5 c.c. blood samples taken for the determinations.

(c) *Procedure.*

The 5 c.c. blood samples are centrifuged for 10 minutes at 3000 revolutions per minute, and the plasma drawn off carefully to avoid contamination with white cells. Exactly 2 c.c. of plasma is diluted with 2 c.c. of 0·9 per cent. NaCl, and 0·5 c.c. of this mixture (corresponding to 0·25 c.c. plasma) is placed in each of four 15 c.c. test-tubes. In case of suspected generalized bone disease, *e. g.* osteitis fibrosa cystica or osteitis deformans (Paget),* 1 c.c. of the plasma diluted 1 : 1 is further diluted by adding 4 c.c. (or even 9 c.c.) of 0·9 per cent. NaCl. (Greater dilution than this is occasionally advisable.) Of the resulting mixture 0·5 c.c., equivalent to 0·05 (or 0·025 c.c.) of the original plasma, is used along with the standard dilution.

To each of the test-tubes 5 c.c. of substrate solution 7 is now added, and the contents mixed. Two (or more if several dilutions of plasma have been made) of these tubes are stoppered with clean rubber stoppers and placed in the water-bath at 38° C. To the other two tubes 2 c.c. of 15 per cent. trichloroacetic acid is added without delay; they are stoppered, mixed and, after 10 minutes, the contents are filtered through two 7 cm. Whatman No. 30, or any other phosphate-free filter-papers. The filtrates are set aside ("zero filtrates"). The other tubes remain in the water-bath for exactly 3 hours; they are then cooled rapidly under the cold-water tap and 2 c.c. of trichloroacetic acid added to each. After standing for 10 minutes the contents of the tubes are filtered through two phosphate-free filter-papers.

From each of the four filtrates exactly 5 c.c. is measured into a clean labelled test-tube and two "standard" tubes, one containing 5 c.c. of standard solution 11 (*a*) and the other 5 c.c. of standard solution 11 (*b*), are made up at the same time.† 4 c.c. of the molybdic acid (solution 9) is then added and finally 1 c.c. of freshly-prepared, dilute $SnCl_2$ solution (solution 10), *stirring whilst adding.*

* In these diseases colours too deep to read with any accuracy are given in the "incubation" tubes unless the plasma is further diluted.

† In case of need, 5 c.c. of standard solution 11 (*c*) may also be used, but it is advisable to employ this deeper coloured standard as little as possible, and by suitable dilution of the original plasma keep the hydrolysis readings between 0·010 and 0·025 mgm. P.

Stirring is most conveniently done by means of a glass rod ending in a small disc, which is moved up and down in the test-tube. The stirring rod should be clean, so that no drops adhere to it, and should be rinsed in distilled water between each stirring. The two solutions should be added to the filtrates with sufficient care to keep the final volume within the limits of $10 \pm 0{\cdot}1$ c.c. The blue colour which develops rapidly in all the six tubes should be read after 3 minutes in any good colorimeter against the nearest of the standard tubes. Errors begin to arise if the colour of the unknown is lighter than, or deeper than that of the standard by more than about 40 per cent. of the latter.

(d) *Calculation.*

The above determinations, as read in the colorimeter, easily give us the amount of P in milligrammes contained in 5 c.c. of the filtrates. (The duplicate determinations should agree within 3 per cent.) The "zero" tube average figure is subtracted from the "incubation" figure, giving, say, a mgm.; a mgm. then represents the amount of inorganic phosphate liberated in 5 c.c. of the filtrate. Since the volume before filtration was 7·5 c.c., the total amount of P liberated during hydrolysis by 0·25 of plasma is $\frac{7{\cdot}5}{5}\,a$ mgm. Therefore 100 c.c. plasma* liberate $600 \times a$ mgm. The phosphatase content of a sample of plasma is defined as the number of mgm. P which would be liberated by 100 c.c. of the plasma under the conditions described above.

A few examples of the type of results obtained using this method are given in Tables I, II and III:

TABLE I.—*Plasma Phosphatase by New Method in Normal Individuals.*

No.	Age.	Sex.	Description.	Enzyme content of plasma. New method.	Older method (Kay, 1930).	Older method × 50.
1	18	F.	Normal	5·8	0·14	7·0
2	19	F.	,,	4·8	0·08	4·0
3	25	M.	,,	4·8	0·10	5·0
4	22	M.	,,	7·9	0·16	8·0
5	28	F.	,,	6·4	0·13	6·5
6	29	F.	,,	6·5	0·12	6·0
7	23	M.	,,	7·4	0·15	7·5
8	21	M.	,,	3·2	0·09	4·5
9	38	M.	,,	6·5	0·12	6·0
10	41	M.	,, (neurasthenia)	7·3	0·15	7·5
		Average		6·06	0·124	6·20

The average for normals of 6·06 new units by the new method is comparable with an average by the older method of 0·124 old units, *i. e.* is about 50 times as great. The new unit is thus about 1/50 of the old unit.

* If any further dilution of plasma was made at the start, as, for example, would be done in case of generalized bone disease, the necessary calculation can easily be introduced here.

TABLE II.—*Plasma Phosphatase by New Method in Various Pathological Conditions, excluding Bone Disease.*

No.	Age.	Sex.	Description.	Enzyme content of plasma. New method.	Older method.	Older method × 50.
11	74	F.	Muscular wasting	4·5	0·11	5·5
12	53	M.	Diabetes	6·5	0·09	4·5
13	46	M.	Gastric neurosis	7·6	0·12	6·0
14	32	M.	Gastric disease (?)	7·0	0·16	8·0
15	29	M.	Hypertension	7·4	0·16	8·0
16	58	M.	Enlarged liver	11·5	0·22	11·0
17	51	M.	Nephritis	7·8	0·17	8·5
18	34	M.	Renal calculus	4·8	0·11	5·5
19	58	F.	Colitis	7·8	0·14	7·0
20	68	M.	Ca. prostate. No secondaries shown by X-ray	6·6	0·11	5·5
21	54	F.	Acute enteritis	11·8	0·25	12·5
22	42	F.	Hæmatemesis	6·7	0·14	7·0
23	43	F.	Rheumatoid arthritis	6·3	0·12	6·0
23	Same individual, one week later			6·3	0·13	6·5
24	42	M.	Arthritis	14·3	0·30	15·0
25	47	M.	Tuberculosis of right knee	7·9	0·16	8·0
26	21	F.	Pulmonary tuberculosis	8·9	0·16	8·0
27	32	F.	,, ,,	11·6	0·25	12·5
28	51	M.	Arthritis	9·4	0·18	9·0
29	25	M.	,,	6·6	0·11	5·5
30	26	M.	,,	10·0	0·19	9·5

TABLE III.—*Plasma Phosphatase by New Method in Various Bone Diseases or Injuries.*

No.	Age.	Sex.	Description.	Enzyme content of plasma. New method.	Older method.	Older method × 50.
31	16	M.	Tuberculosis of thoracic vertebræ	14·7	0·33	16·5
32	..	M.	Infective arthritis with bone atrophy	7·5	0·17	8·5
33	20	M.	Scoliosis	9·5	0·21	10·5
34	38	M.	Fracture of radius and ulna	6·8	0·14	7·0
35	21	M.	Fracture of right tibia	4·9	0·14	7·0
36	16	M.	Fracture of femur (adolescent goitre)	6·1	0·18	9·0
37	17	M.	Fracture of right astragalus	5·9	0·13	6·5
38	20	M.	Fracture of upper iliac crest	10·4	0·30	15·0
39	35	M.	Fracture of 3rd cervical vertebra	5·6	0·13	6·5
40	24	F.	Osteogenic sarcoma	11·5	0·23	11·5
41	32	M.	Osteomyelitis, lower jaw	5·8	0·14	7·0
42	18	M.	Old fracture of humerus with mal-union	13·7	0·31	15·5
43	46	M.	Cervical lymphadenitis	5·9	0·13	6·5
44	70	F.	Paget's disease	64·1	2·01	100·5
45	..	F.	,, ,,	160·0	4·53	226·0
46	35	M.	Osteitis fibrosa cystica	172·0	>1·30	>65·0
46	Same individual 3 months later (no operation as yet)			172·0	5·8	290·0

Autolysis Correction.

Under the conditions just described the autolysis during the incubation period, either of the phosphoric esters or the lipins of the plasma, is so small that it may be completely ignored.

While the method just described was being tested with a variety of human plasmas from healthy and diseased subjects, a short abstract was published by Bodansky (1931) of a somewhat similar method which he had recently developed for the determination of phosphatase in the plasma of guinea-pigs. As buffer he used sodium di-ethyl barbiturate (veronal), which Michaelis (1930) has shown to be an excellent buffer within the pH range of optimal activity of animal phosphatase. We had, ourselves, previously tried Michaelis's buffer, and had found that for our purpose it had no advantage over glycine, besides being much more expensive, and its use occasionally complicated by the precipitation of di-ethyl barbituric acid when acid filtrates containing veronal were allowed to stand.

SUMMARY.

A method for the determination of plasma phosphatase is described, suitable for use in the clinical laboratory. It requires only small quantities of plasma (2 c.c. is ample; determinations may be done on 1 c.c. or even less), and may be completed, from the drawing of blood to the working out of the final value, in 4 hours. Other than the usual laboratory glassware, the only apparatus required is a colorimeter.

Our thanks are due to Dr. E. J. Maltby, of the Department of Medicine, and to Dr. W. G. Carscadden, of the Department of Surgery, University of Toronto, for their kind co-operation in providing us with most of our clinical material.

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THE STANDARDIZATION OF ANTIDYSENTERIC SERUM (SHIGA).

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THE increasing importance for a uniform standard method for estimating the potency of antidysenteric serum has been in the minds of different government laboratories, and many methods have been suggested.

The National Institute of Health in Washington sends a control standard serum, and requires that the agglutination titre of the test serum be as good or better than the standard serum, according to the requirements of the U.S. Public Health Service. This method of testing takes into consideration only the agglutinins, and to our knowledge they bear no relation to the antitoxic properties of the serum.

The International Serum Conference held in 1926, on the invitation of the Health Section of the League of Nations, provisionally adopted as one unit of antidysenteric serum (Shiga) the quantity of the Copenhagen (dry) standard contained in 1/200th part of a cubic centimetre of a 1 per cent. solution. This unit has been adopted in England in accordance with the Regulations made under the Therapeutic Substances Act. The standard serum was prepared by the State Serum Institute of Copenhagen, and is kept at the National Institute for Medical Research, London. This serum is kept dry over P_2O_5 at constant weight and sealed in dry nitrogen, according to British Regulations.

The standard solution is prepared by dissolving the dry serum in a mixture of 2 parts of glycerol with 1 part of physiological saline. The solution is so adjusted that it contains exactly 1 per cent. of the dry serum standard, so that 1/200 c.c. of the standard serum solution represents 0·05 mgm. of the dry standard and in turn 1 unit of antidysenteric serum (Shiga).

To make the test, a suitable toxin must be prepared. We have found the method of Okell and Blake (1930) very satisfactory, giving a good yield as well as a potent toxin. With this toxin the " test dose " has to be determined, using the standard serum solution in 1/200 c.c. and graded doses of the toxin. This mixture is injected into mice intravenously, giving the desired results. With our own lot of toxin we have found that 0·2 mgm. is our " test dose." Five lots of antiserum have been tested by this method, and the results of the tests are given in Table I.

In studying the polysaccharides of many different organisms, we found (as also did Morgan, 1931) that *B. dysenteriæ* (Shiga) has a specific carbohydrate which gives a precipitate with a specific immune antiserum. By applying the same method that we used in the standardization of antipneumococcus serum (Zozaya, Boyer and Clark, 1930), we have found that antidysenteric serum (Shiga) can be standardized equally well by the polysaccharide

TABLE I.—*Potency Test of Antidysenteric Serum by the British Standard Method.*

Serum.	Serum dose (c.c.).	Test dose toxin 0 (mgm.).	Number of mice dying in 7 days.	Number of mice surviving in 7 days.	Percentage of mice surviving.	Number of mice found dead at—							Unit value.
						18 hrs.	24 hrs.	2 days.	3 days.	4 days.	5 days.	6 days.	7 days.	
British standard D.S. 7	1/150	0·2	0	5	100	(1)	0	0	0	0	0	0	0	200
	1/200	0·2	2	4	66	0	1	1	0	0	0	0	0	
	1/300	0·2	4	1	20	(1)	0	1	1	1	0	1	0	
	1/400	0·2	6	0	0	0	0	1	3	2	..	..	..	
Antidys. ser. poly. 10143	1/500	0·2	4	0	0	0	1	2	1	..	..	..	..	< 500
Ditto 91946	1/500	0·2	1	5	83	0	0	1	0	0	0	0	0	> 500
Ditto 91945	1/400	0·2	0	6	100	0	0	0	0	0	0	0	0	600
	1/500	0·2	0	6	100	0	0	0	0	0	0	0	0	
	1/600	0·2	0	6	100	0	0	0	0	0	0	0	0	
	1/700	0·2	4	2	33	0	1	0	0	1	0	0	2	
Antidys. ser. conc. 22140	1/1000	0·2	0	6	100	0	0	0	0	0	0	0	0	1200
	1/1200	0·2	0	6	100	0	0	0	0	0	0	0	0	
	1/1600	0·2	3	2	40	0	0	0	0	1	0	0	2	
	1/2000	0·2	4	1	20	(1)	1	1	0	0	1	1	0	
Antidys. ser. 20972 (original of conc. 22140)	1/200	0·2	1	5	83	0	0	1	0	0	0	0	0	400
	1/400	0·2	1	5	83	0	1	0	0	0	0	0	0	
	1/500	0·2	3	2	40	0	0	0	0	1	2	0	0	
	1/800	0·2	5	1	17	0	0	1	0	4	0	0	0	

precipitating test, comparing the titration of the test serum with that of the standard serum solution or its equivalent.*

The preparation of the polysaccharide followed similar methods to those suggested by Heidelberger and Avery (1924) for the pneumococcus with some slight modification. The amount of polysaccharide that we have used was 0·2 c.c. of a 1/1000 dilution, with equal amounts of serum of different dilutions. The tubes were placed in the water-bath at 37° C. for 2 hours, then left in the ice-box overnight, when they were read. Since we found the standard serum solution unsatisfactory for comparison, we took one of our lots of antiserum (No. 91945), which we have employed in several tests and found to be 600 units, diluted it with salt solution in the ratio of 1/3 and determined the precipitin titre of this sample. The results are shown in Table II. From this we calculated that the highest dilution of the serum which gave a good (2+) precipitate in the overnight reading, multiplied by 20, would give the number of units in the test serum. All recorded tests are overnight readings.

TABLE II.—*Titration of Serum* 91945 *Diluted* 1 : 3 *to Compare with the British Standard Serum Solution—Polysaccharide* 1/1000.

Final dilution.	1/2.	1/4.	1/6.	1/8.	1/10.	1/12.	Units from mouse test.	Factor.
Serum 91945, 1 : 3	4	4	4	3	2	0	200	$\frac{200}{1-10} = 20$

4, 3, 2, 0 refer to the degrees of precipitation, 4 being complete.

From these results the same five samples of antidysenteric serum (Shiga), shown in Table I and others, could be titrated, as shown in Table III, with the calculated number of units as well as the actual units found by the mouse method.

TABLE III.—*Titration of Antidysenteric Serum* (*Shiga*) *with Homologous Polysaccharide* 1/100.

Serum.	Final dilution of serum.											Mouse test units.	Calculated units.
	1/2.	1/4.	1/8.	1/12.	1/16.	1/24.	1/32.	1/40.	1/48.	1/56.	1/64.	1/72.	
91945	4	4	4	4	4	3	2	0	0	0	0	0	600	640
22140	..	..	4	4	4	4	4	4	4	3	2	0	1200	1280
D.S. 7	..	..	..	..	..	..	..	..	..	..	..	..	200	..
20972	4	4	4	4	3	2	0	0	0	0	0	0	400	480
91946	4	4	4	4	4	3	2	0	0	0	0	0	500+	640
10143	4	1	0	0	0	0	0	0	0	0	0	0	<50	<80
9049	0	0	0	0	0	0	0	0	0	0	0	0	..	0
9026	3	2	0	0	0	0	0	0	0	0	0	0	<50	<80
91943	4	4	4	3	2	1	0	0	0	0	0	0	200 to 600	320
91944	4	4	4	4	3	2	1	0	0	0	0	0	600	480
16966	2	2	1	0	0	0	0	0	0	0	0	0	<50	<80
92216	4	4	4	2	1	0	0	0	0	0	0	0	400 to 600	240
91947	4	4	4	4	4	2	0	0	0	0	0	0	400	480
91942	4	4	4	4	4	2	0	0	0	0	0	0	400	480

* Glycerol used in the dilution of the standard serum solution interferes somewhat with the polysaccharide precipitin test.

TABLE IV.—*Potency Test of Antidysenteric Serum Compared with the British Standard Method.*

Serum.	Serum dose (c.c.).	Test dose Shiga toxin 0 (mgm.).	Number of mice dying in 7 days.	Number of mice surviving in 7 days.	Percentage of mice surviving.	Number of mice found dead at—							Unit value.
						18 hrs.	24 hrs.	2 days.	3 days.	4 days.	5 days.	6 days.	7 days.	
British standard	1/150	0·2	1	5	83	0	1	0	0	0	0	0	0	
D.S. 7	1/200	0·2	3	3	50	0	1	0	0	0	1	1	0	200
	1/300	0·2	6	0	0	0	1	1	2	1	1	..	..	
	1/400	0·2	6	0	0	0	1	1	3	0	1	..	..	
Ditto	1/150	0·2	5	1	17	0	3	0	2	0	0	0	0	
D.S. 8	1/200	0·2	5	1	17	0	2	1	2	0	0	0	0	< 150
	1/300	0·2	6	0	0	0	3	0	1	2	..	..	..	
	1/400	0·2	6	0	0	0	0	3	3	..	..	..	..	
Antidys. ser. poly.	1/50	0·2	4	2	33	0	2	1	0	1	0	0	0	
10143	1/100	0·2	6	0	0	0	0	3	3	..	..	..	..	<50
	1/200	0·2	6	0	0	0	1	3	2	..	..	..	..	
Ditto	1/400	0·2	2	4	66	0	2	0	0	0	0	0	0	
91946	1/600	0·2	2	4	66	0	0	0	1	0	0	1	0	600
	1/800	0·2	5	1	17	0	0	2	0	2	1	0	0	
Ditto	1/400	0·2	2	4	66	0	2	0	0	0	0	0	0	
91942	1/600	0·2	5	1	17	0	0	0	2	0	1	2	0	400
	1/800	0·2	5	1	17	0	2	1	1	0	1	0	0	
Ditto	1/200	0·2	1	5	83	0	1	0	0	0	0	0	0	200
91943	1/400	0·2	3	3	50	0	1	1	1	0	0	0	0	to
	1/600	0·2	3	3	50	0	0	0	2	0	0	1	0	600
Ditto	1/400	0·2	3	3	50	0	3	0	0	0	0	0	0	
91944	1/600	0·2	2	4	66	0	1	0	1	0	0	0	0	600
	1/800	0·2	4	2	33	0	0	0	0	0	0	1	3	
Ditto	1/400	0·2	2	3	60	0	0	1	1	0	0	0	0	
91947	1/600	0·2	6	0	0	0	2	1	1	0	1	1	..	400
	1/800	0·2	5	1	17	0	2	1	0	0	1	1	0	
Ditto	1/200	0·2	1	5	83	0	1	0	0	0	0	0	0	400
92216	1/400	0·2	1	5	83	0	1	0	0	0	0	0	0	to
	1/600	0·2	3	3	50	0	3	0	0	0	0	0	0	600
Ditto	1/50	0·2	6	0	0	0	0	3	1	2	..	..	..	
9026	1/100	0·2	6	0	0	0	0	6	..	..	..	..	..	< 50
Ditto	1/50	0·2	6	0	0	0	0	3	3	..	..	..	..	
16966	1/100	0·2	6	0	0	0	0	3	2	1	..	..	..	< 50

Lot No. 10143, which in the mouse test did not give an end-point, had to be repeated. Lot No. 91946, which in the mouse test gave 500 units and in the polysaccharide precipitin test gave 640, was also repeated. The findings of these sera are recorded in Table IV, where a better agreement of this test with the polysaccharide precipitin results may be observed.

Concerning sera 91943 and 92216, the mouse test is indefinite on account of the percentage of mice surviving in the standard serum. If we accept 50 per cent. survivals as 200 units, then serum 91943 will be 400 or 600 units, and serum 92216 will be 600 units. If we accept 70 per cent. survivals or more in these two tests, then we will get, in the mouse test, over 200 units in 91943 and 400 units in 92216. Here we have a clear example of the unavoidable variations associated with animal-protection tests. The polysaccharide test gives clear-cut results that can easily be repeated.

DISCUSSION.

The use of the polysaccharide method for determining the potency of antipneumococcus serum has worked so very satisfactorily, not only in " straight " serum, but also in the concentrated lots, that we have looked for further application of this method to other serums. The methods of standardization so far employed have shown many irregularities when used in different laboratories due to minor variations in technique and to variations in susceptibility of the test animals. The polysaccharide precipitin method gives more constant results than any method so far used, for the carbohydrate, even if it is somewhat impure, gives similar results at all times. The test has the advantage of being very simple. Presumably the other types of antidysenteric serums (Hiss, Flexner and others), which are less commonly used, can be standardized by this same method, using their specific carbohydrate in each case.

CONCLUSION.

A practical method is given for the standardization of antidysenteric serum (Shiga) by the specific polysaccharide precipitin test.

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CROSS-IMMUNITY BETWEEN SOUTH AFRICAN TYPHUS AND TICK-BITE FEVER.

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SOUTH AFRICAN TYPHUS.

SOUTH African typhus may ultimately be found not to be a homogeneous pathological entity, but so far there are no grounds not to regard it as such. Minor clinical variations and failure to find lice in individual cases do not warrant the creation of a separate group, as suggested by Scroggie (1931). South African typhus clinically resembles European typhus, but is on the whole milder, and the mortality is lower. It forms agglutinins against *X* 19 (Pirie, 1922), and also against *X* 2, but not against *X* Kingsbury (Pijper and Dau, 1931, *b*). The titre is usually much lower than in European typhus

Our first attempt at transmitting the disease from a patient to guinea-pigs succeeded, and we have kept this strain of virus alive ever since by serial transmissions. We use brain suspensions and intraperitoneal inoculation. This virus has now reached its twenty-eighth guinea-pig passage. Post-mortems show the usual changes. We have now used this strain for cross-immunity experiments with tick-bite fever. Section 1 of Fig. 1, on the left, gives a typical fever curve of our animals. On the right the identical animal is shown not to react to a second inoculation, whilst a control animal, inoculated at the same time with a similar quantity of the same brain suspension, shows the typical fever reaction. As this immunity experiment has been repeated four times, always with results as depicted in section 1, it may be stated that our strain of typhus virus produces immunity in guinea-pigs against itself.

TICK-BITE FEVER.

We regard tick-bite fever as a "typhus-like disease" on the following grounds: Clinical similarity (Pijper and Dau, 1931, *a*), agglutination of all three *X*-strains by patients (Pijper and Dau, 1930, *b*), transmissibility of virus to guinea-pigs in series with production of fever curves resembling those of typhus (Pijper and Dau, 1930, *a*), and formation of nodules in the brain of infected guinea-pigs (Pijper and Dau, 1930, *b*). The characteristic primary tick-bite sore differentiates the disease from typhus.

Intraperitoneal inoculation of patients' blood into guinea-pigs does not always produce a typical fever curve. In many instances we only got irregular

rises of temperature, or no temperatures at all. In such cases repeated guinea-pig passages sometimes resulted in more typical fever curves. In a few

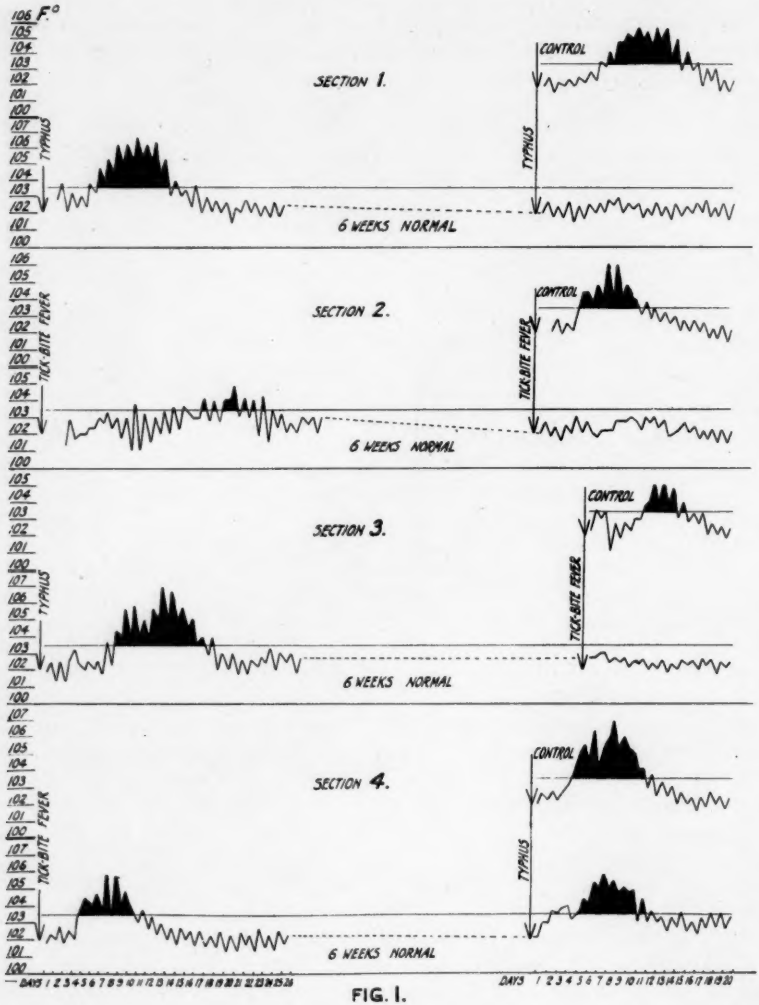

FIG. I.

instances we got a typical fever curve from the start. Serial tansmissions by means of brain suspensions then as a rule succeeded quite well. In one instance we reached a fifth passage. At the moment we have a strain which

has reached its tenth guinea-pig passage, producing typical fever curves all the time. This strain has been used principally for the cross-immunity experiments.

Section 2 of Fig. 1 shows that a guinea-pig which has shown a good, but not quite typical, fever reaction in response to a first inoculation of tick-bite fever material is immune against a second inoculation six weeks later, whilst the control animal reacted with a typical curve. This immunity experiment has been repeated four times, various strains of virus being used, but chiefly the particularly good strain mentioned above. We may therefore take it that, as in typhus, the virus of tick-bite fever produces immunity against itself in guinea-pigs.

CROSS-IMMUNITY.

Section 3 of Fig. 1 shows that a guinea-pig which has produced a typical fever curve after inoculation with typhus virus is immune against a dose of tick-bite fever six weeks later, whilst a control animal, infected with a similar dose, shows a typical fever curve.

In a similar way, section 4 of Fig. 1 illustrates that tick-bite fever does not immunize a guinea-pig against typhus virus.

Both these cross-immunity experiments have been repeated four times, with exactly similar results.

CONCLUSION.

The virus of South African typhus confers immunity in guinea-pigs against tick-bite fever virus. Tick-bite fever virus, however, does not confer immunity in guinea-pigs against South African typhus.

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FILTRATION OF VACCINIA VIRUS THROUGH GRADOCOL MEMBRANES.

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THE filterability of vaccinia virus has been studied by a refined method of graded filtration, using the new series of gradocol collodion membranes described by Elford (1931, *a*). Knowledge of the filtration properties of this virus has been derived in the past mainly from experiments with filter-candles, and the recorded evidence in the literature (Blaxall, 1930) is very confusing. While some workers have claimed the virus to be filterable through bacteria-tight filters, others have failed to demonstrate its presence in filtrates. It is abundantly clear that a more refined and accurately controlled filtration technique is required in this problem, together with a better understanding of the determining factors in the processes concerned. Gradocol membranes have already been found well suited for virus investigation in studies upon infectious Ectromelia (Barnard and Elford, 1931) and foot and mouth disease (Galloway and Elford, 1931). The present study with vaccinia virus provides further evidence of their usefulness in enabling the size of the virus particles to be estimated. Very recently Bechhold and Schlesinger (1931) have reported filtration experiments using membrane and cellafilters (essentially collodion membranes) prepared commercially by a Göttingen firm. Their estimation of the size of vaccinia virus from filtration data leads them to the value 0·2μ, and this is supplemented by centrifugal analysis, giving a figure of 0·21–0·23μ. We shall refer to this work later in its relation to the results of our own study. It may be mentioned that 0·25μ is the figure generally agreed upon as the size of a Paschen body.

Filtration Technique.

Reference should be made to the previous paper (Elford, 1931, *a*) for the details of preparation and properties of gradocol membranes. They are graded in terms of their average pore size values, calculated according to Poiseuille's law from measurements of the rate of flow of water through membranes under known conditions. The maximum pore size given by critical air-pressure tests and the average pore size are found to run parallel as far as the air-pressure test is applicable, the maximum being approximately twice the average value. This figure contrasts favourably with that found for the acetic acid collodion membranes which have been chiefly used in the past in the study of viruses; with them the maximum pore size is usually 10–20 times the average. The adoption of the average pore size as basis of

gradation enables a continuous standardization of the most permeable to the least permeable members of the series to be made.

The form of ultra-filtration apparatus and the technique of sterilizing and assembling this have been precisely as described in previous studies (Elford, 1931, *a*). Filtration is conducted under a positive applied pressure of air or nitrogen.

Technique of Preparing and Testing Virus.

The virus used was a testicular strain of Levaditi's neurovaccine. Rabbits were inoculated intratesticularly and killed after 3—occasionally 4—days. The aseptically removed testes were minced; quantities measured with a tumour syringe were ground, as recommended by Ward (1929), with powdered pyrex glass and made up to an emulsion of the required strength, usually 1 or 2½ per cent., with ox-heart broth. In view of Ward's difficulty in filtering through candles virus which had been stored in the cold, we used fresh virus for each experiment. On two occasions, however (Experiments 5 and 8 in Table I) we used old frozen virus, and did not find that the virus was strikingly less filterable than when freshly prepared. We did not, however, investigate this question further. The emulsion was filtered through sand and paper-pulp to remove coarse tissue particles, and the filtrate thus provided again filtered through a permeable membrane to yield a clear sterile stock filtrate for subsequent work through less permeable membranes.

Filtrates were tested by making serial dilutions in saline; 0·1 c.c. quantities were inoculated intradermally into the epilated skins of rabbits. Duplicate rabbits were used in nearly all experiments. Where Table I states that the titre of a given filtrate was, say, 10^3–10^4, it is implied that one rabbit gave a take with 0·1 c.c. of at most a 1 : 10^3 dilution, while the other animal was positive at 1 : 10^4.

Results.

The results are shown in Table I. It is clear that, other conditions being equal, the tightness of the membrane which the virus can pass depends upon the titre of the material filtered. Thus virus which did not take in a higher dilution than 1 : 1000 was held up by a 0·6μ membrane; while virus active at 1 : 10^5 could pass through a 0·3μ membrane. In the only experiment in which we obtained a clear paper pulp and sand filtrate having a titre of over 1 : 10^6, a filtrate through a 0·26μ membrane was active. As we could not repeat this result, and as this filtrate was of suspiciously high activity, we do not feel disposed to stress this isolated finding. The dependence of filtrability on titre is strikingly shown in experiment 10, where a 1μ filtrate having a titre of 10^4–10^5 passed a 0·3μ membrane (test on rabbits 76 and 77). The same 1μ filtrate, when diluted ten or a hundred times, failed to pass a 0·5μ filter (rabbits 78, 79, 80, 82). These findings are easy to understand if we take account of the fact that collodion membranes have a definite power of absorbing the virus, and only when this capacity is satisfied can the virus pass. Doubtless virus having a low titre would have passed the tighter membranes if we could have filtered large enough quantities. In practice it was found that with the tightest membranes filtration was very slow after 10 c.c. had

TABLE I.—*Effect of Titre on Filterability through Membranes of Various Grades.*

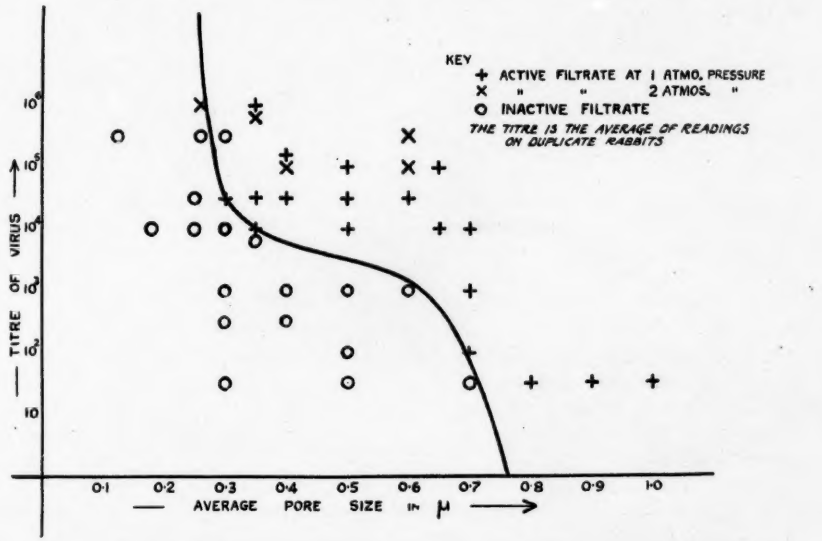
Experiment.	Rabbit No.	Titre of filtrates through membranes of given grade.				Remarks.
		Unfiltrable.	P.P.S.	0·65μ.	0·30μ.	0·65μ. 0·30μ.
1	1	..	10^4	..	..	p = 76 cm. Hg. 76 cm. Hg.
	2	10^5	..	..	0	Emulsion = 1·0%.
	3	..	10^5	10^4	..	
	4	10^6	..	10^3	0	
		0·65μ.	0·40μ_1.	0·40μ_2.	0·30μ.	0·65μ. 0·40μ_1. 0·40μ_2. 0·30μ.
2	6	10^2	0	..	..	p = 76 cm. Hg. 76 cm. Hg. 2 atms. 2 atms.
	7	10^3	..	0	..	Emulsion = 1·0%.
	8	..	0	..	0	
	9	10^3	..	0	0	
		P.P.S.	0·50μ_1.	0·50μ_2.	0·40μ.	0·50μ_1. 0·50μ_2. 0·40μ.
3	11	10^5	10^4	..	..	p = 2 atms. 76 cm. Hg. 3 atms.
	12	10^5	10^3	..	..	Emulsion = 1·0%.
	13	..	..	10^2	10^4	
	14	10^5	..	10^2	10^2	
		P.P.S.	0·40μ.	0·35μ.	0·26μ.	0·40μ. 0·35μ. 0·26μ.
4	16	10^4	..	0	..	p = 76 cm. Hg. 76 cm. Hg. 76 cm. Hg.
	17	10^5	..	..	0	Emulsion = 1·0%.
	18	..	10^2	10	..	
	19	..	10^2	..	0	
		P.P.S.	0·65μ.	0·40μ.		0·65μ. 0·40μ.
5	20	10^5	..	..	..	p = 76 cm. Hg. 76 cm. Hg.
	21	10^5	10^3	..	..	Emulsion = 1·0% frozen virus.
	22	..	10^3	10^2	..	
	23	..	..	10^2	..	
		P.P.S.	1·0μ.	0·35μ.	0·26μ ?	1·0μ. 0·35μ_1. 0·35μ_2. 0·26μ.
6	29	10^6	..	10^3 } 1.	..	p = 20 cm. Hg. 76 cm. Hg. 2 atms. 2 atms.
	30	10^6	..	10^3 }	..	Emulsion = 4·0%.
	31	..	10^6	10^3 } 2.	10^3	
	32	..	10^6	10^3 }	10^3	
		1·0μ.	0·26μ.	0·125μ.		1·0μ. 0·26μ. 0·125μ.
7	34	10^5	0	0	..	p = 20 cm. Hg. 2 atms. 2 atms.
	35	10^6	0	0	..	Emulsion = 5·0%.
		P.P.S.	0·60μ_1.	0·60μ_2.		0·60μ_1. 0·60μ_2.
8	36	10^4	10^3	..	..	p = 76 cm. Hg. 76 cm. Hg.
	37	10^5	..	0	..	Emulsion = 2·5% frozen virus.
	38	..	10^3	0	..	
		P.P.S.	0·60μ_1.	0·60μ_2.		0·60μ_1. 0·60μ_2.
9	42	10^6	10^5	..	..	p = 2 atms. 2 atms.
	43	10^5	..	10^5	..	Emulsion = 2·5%.
	44	..	10^5	10^4	..	
		1·0μ.	0·70μ.	0·50μ.	0·30μ.	1·0μ. 0·70μ. 0·50μ. 0·30μ.
10	76	10^4–10^5	..	10	..	p = All at 76 cm. Hg.
	77	..	10^4	..	Undil.	Emulsion = 2·5%.
	78*	10^2	10	0	0	
	79	..	10^2	0	0	* 1 : 10 dilution of 1·0μ filtrate.
	80†	10^4	..	0	..	† 1 : 100 ,, ,,
	81	..	10^2	..	0	
		P.P.S.	1·0μ.	0·90μ.	0·80μ.	1·0μ. 0·90μ. 0·80μ.
11	101	10^2	10	10	10	p = All at 76 cm. Hg.
	102	10	10	10	10	Emulsion = 2·5%.
		1·0μ.	0·30μ.	0·25μ.	0·18μ.	1·0μ. 0·30μ. 0·25μ. 0·18μ.
12	109	10^4	0	0	0	p = All at 76 cm. Hg.
	110	10^4	0	0	0	Emulsion = 2·5%.

Volume filtered in all experiments = 10 c.c. per sq. cm. of membrane except in preparation of stock filtrates through coarse membranes, when naturally larger volumes were filtered. P.P.S. = paper pulp and sand filter.

passed a membrane of 1 sq. cm. filtering area. In order to obtain uniform results we usually filtered this volume throughout—or correspondingly larger volumes for membranes of larger surface area.

The absorbing capacity of the membranes is illustrated by an experiment in which some virus active at 1 : 10^3 was filtered through a 0·4μ membrane. The filtrate coming through was collected in small samples, which were tested separately. Before any activity in the filtrate was detected at all, 19 c.c. had to be filtered ; after that increasing quantities came through.

The relation of titre to filterability is shown graphically in the chart. The curve is only drawn very tentatively. Theoretical considerations would,



however, suggest for it a shape such as we have given it. It would be expected to be steep where the size of the pore is large in relation to that of the diameter of the virus, for under these conditions the titre of the virus will not greatly affect its filtration. Possibly the virus particles are not of strictly uniform size ; such a factor may have some influence in determining the shape of the curve. As the true end-point for filtration is approached the curve tends to become perpendicular, since no increase in titre will avail to force the virus through a pore too small for it. Only in the intermediate zone is the slope more gradual, where the filterability of the virus is determined largely by its titre, owing to the large part played by physical forces hindering the passage of virus.

Effect of pH on Filtration of Virus.

An experiment was conducted to determine whether or not pH had any marked influence on the filtration. Infected rabbit testis ground up with

pyrex glass powder was emulsified in a given volume of broth, the pH of which had been adjusted to desired values by the addition of HCl or NaOH. After filtration through a paper pulp and sand filter in the usual way, the filtrate so obtained was again filtered through a 1·0μ membrane. This filtrate served in each case as stock for subsequent filtration through a 0·49μ membrane. The actual pH values and experimental data are given in Table II.

TABLE II.—*Effect of pH on Filterability.*

pH of emulsion.	Readings on duplicate rabbits. Titre of stock 1·0μ filtrate.	Titre 0·49μ filtrate.	Remarks.
8·2	10^4	10^3	Filtration pressure = 76 cm. Hg.
	10^5	10^3	Volume filtered = 25 c.c.; membrane area = 2·5 sq. cm.
7·4	10^4	10^2	Order of rate of filtration 6·0>7·4>8·2.
	10^4	10	
6·0	10^4	0	Order of opalescence of stock filtrate 8·2>7·4>6·0
	10^4	undiluted only	

The experiment indicates that filtration is favoured on the alkaline side of neutrality, for although the rate of filtration was greatest on the acid side, coinciding with maximum clarity of the 1·0μ filtrate, yet the virus was found in highest concentration in the pH 8·2 filtrate. However, as all our experiments had been conducted at pH 7·6, this value was considered sufficiently near the optimum value for stability and filterability of virus for all practical purposes.

Effect of Immune Serum on Filterability.

In unpublished experiments with a coli bacteriophage we have found that a quantity of immune serum insufficient to neutralize the phage will prevent it from passing a candle or a membrane which it can normally pass quite readily. It seemed possible that antivaccinial serum might similarly alter the physical properties of vaccinia virus, especially in view of the claim (Ledingham, 1931) that Paschen bodies can be specifically agglutinated by dilute immune serum. Experiments planned to test this idea must naturally take account of the effect of the titre of virus on its filterability; for a virus-immune-serum mixture might give an inactive filtrate simply because of the drop in the titre of the virus and not because of any change in its physical properties. We accordingly mixed a 0·7μ filtrate having a titre of $1:10^5$ with an equal volume of undiluted immune serum. After two hours' contact at 37° C., the titre of the virus was found to be reduced fifty-fold. Yet 5 c.c. of this immune-serum-virus mixture passed another 0·7μ membrane with undiminished titre. Tighter membranes were not used, as virus with a titre as low as that of this mixture could not have been expected to pass them under the conditions of our experiment. We thus confirmed results previously obtained in similar experiments

in which Berkefeld candles were used: there was no evidence that specific antibodies in sub-neutralizing doses affected the filterability of vaccinia.

DISCUSSION.

The effect of titre on filterability has been stressed because of its importance where the sizes of different viruses are being estimated by methods involving filtration. For example, we have succeeded in one out of three attempts in filtering the Rous sarcoma virus through a 0·25μ membrane—approximately the end-point for vaccinia. Yet it would be most unwise to deduce that the Rous virus and vaccinia were of the same size, for the Rous filtrate used is unlikely to have had a titre of more than 1 : 100—or at the very most 1 : 1000. That the Rous virus is indeed smaller than vaccinia is suggested by the findings of other workers (Zinsser and Tang, 1927; Mendelsohn, Clifton and Lewis, 1931), though it is true that they used acetic collodion membranes, to the defects of which we have alluded earlier.

Probable Size of Virus.

The course adopted in estimating the minimum size of particles just retained under the most favourable conditions for filtration by various grades of gradocol membranes has been discussed in a paper on the mechanism of filtration (Elford, 1931, *b*). The relationship between particle size and the average pore size of the limiting grade of membrane for filterability is found to be as follows:

Average pore size of membrane.	Particle size expressed as a fraction of the membrane pore size.
1·0–0·5μ	0·75–1·0
0·5–0·1μ	0·5–0·75
less than 0·1μ	0·33–0·5

On this basis the following estimations are made:

	Limiting membrane.	Particle size.
B. prodigiosus	0·75μ	0·5–0·75μ
Bovine pleuro-pneumonia, smallest phase	0·2μ	0·1–0·15μ
Infectious ectromelia	0·2μ	0·1–0·15μ
Bacteriophage (coli)	60μμ	20–30μμ

The permeability end-point for vaccinia virus has been found in the present study to be 0·25μ. Therefore on the above basis of estimation the size of the smallest virus elements may be given as 0·125–0·175μ. Our estimation is thus somewhat lower than that of Bechhold and Schlesinger, but one would expect the centrifugal analysis to give an average figure probably on the high side in relation to the size of the most elementary units.

The figure given in the above table for the size of infectious ectromelia virus, 0·1–0·15μ, has been confirmed by Barnard and Elford (1931) by a direct optical method, in which images of the virus particles have been obtained by the method of ultraviolet-light photography. Barnard calculates the value 0·14μ

for the size of the virus. This excellent agreement provides good justification for the method used in interpreting the filtration data.

CONCLUSIONS.

1. Filtration experiments through gradocol membranes suggest that vaccinia virus particles have a diameter of 0·125μ to 0·175μ.

2. When other conditions are equal the titre of the virus filtered plays a large part in determining the limiting pore size which gives an active filtrate.

3. Immune serum in quantity insufficient to neutralize the virus completely had no demonstrable effect on its filterability.

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SOME OBSERVATIONS ON THE AGGLUTINATION OF *V. CHOLERÆ.*

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THE serological reactions, whether agglutination, absorption of agglutinins or bacteriolysis, form the final tests to differentiate between *V. cholerœ* and the closely allied vibrios.

The agglutination test is used the most extensively because of its comparative simplicity for the purpose of identification.

Douglas (1921) examined nine strains of *V. cholerœ* from widely different sources; by agglutination and agglutinin absorption they formed one serological group. He, however, worked with formolized cultures which have

been shown to be unsuitable for the demonstration of somatic agglutination. The same worker showed that some strains of paracholera will agglutinate, under certain conditions, with cholera antiserum, but will fail to absorb agglutinins.

Nobechi (1923), and later Ohtsubo, Takano and Inouye (1926) maintained that there were three serological races of *V. cholerœ*.

Weil and Felix (1920) concluded that the *V. cholerœ* possessed an antigen which is thermostable at 100° C., and that they did not possess any thermolabile antigen. The same conclusions were arrived at by Brutsaert (1924).

Balteanu (1926), however, was able to demonstrate the presence of a thermolabile antigen in addition to the thermostable one in *V. cholerœ*.

The present investigation was undertaken—

i. To confirm the presence of a thermolabile antigen in *V. cholerœ*.

ii. To determine whether there are races of *V. cholerœ* differing in their somatic antigens.

iii. To ascertain whether the cross-agglutination which takes place between *V. cholerœ* and the closely allied vibrios is due to a factor or factors connected with the thermolabile or thermostable antigens or both of them.

Cultures.—Twenty-two strains of true cholera vibrios, together with six vibrios labelled El Tor, three paracholera vibrios and twenty-four cholera-like vibrios were utilized in this investigation. These strains were collected from the Lister Institute, London, from the Pasteur Institute, Paris, and the Hygiene Institute, Cairo.

Preparation of sera.—1. Two sera were prepared against *V. cholerœ* strains Jerusalem and Pottevin. Saline emulsions of agar cultures heated to 58° C. for 30 minutes were injected intravenously into rabbits at 6 days' intervals.

2. Three sera were prepared against *V. cholerœ* strains Jerusalem, Bagdad and Pottevin. Saline suspensions were heated to 100° C. for 2 hours and were injected into rabbits.

3. A flagellar suspension was prepared according to the technique described by Orcutt (1924) and adopted by Balteanu (1926). A 24-hour Roux bottle agar culture was suspended in 50 c.c. saline, shaken for 2 hours and then centrifuged for 4 hours at 6000 r.p.m. The supernatant fluid, which is perfectly clear, was injected subcutaneously into rabbits in doses of 1, 2 and 3 c.c. at 6 days' interval.

4. A serum was prepared against suspensions of *V. El Tor* A.T.C.C. 582 heated to 58° C. for 30 minutes, using a technique similar to that described under (1).

5. A serum was prepared against the cholera-like vibrio G.L. sewage heated to 58° C. for 30 minutes.

Technique.—(1) Agglutination tests were done macroscopically in small tubes ; 0·5 c.c. suspension of suitable opacity and 0·5 c.c. of the various serum dilutions were incubated in a water-bath at 55° C.

(2) Thick saline emulsions from 20-hour agar cultures were used. One volume of this emulsion was added to one volume of the serum to be absorbed, diluted 1 : 25 and incubated at 37° C. for 2 hours. Another volume of emulsion was then added, and the mixture kept in the ice-chest overnight.

PART I.

The first part of this work is mainly a repetition of Balteanu's work.

The claim for the presence of a thermolabile antigenic substance in the flagellar emulsion, obtained according to the technique described, was fully verified.

Sera prepared by injecting rabbits with vibrios heated to 58° C. for 30 minutes agglutinated this flagellar emulsion rapidly in the form of large woolly flocculi.

When this flagellar emulsion was heated to 100° C. for an hour, it ceased to agglutinate with the same serum.

The injection subcutaneously of this flagellar emulsion gave rise to a serum which agglutinated with living or formolized cultures rapidly in the form of large woolly flocculi, but failed to agglutinate with cultures heated to 100° C. for 2 hours.

Living and formolized cholera vibrios agglutinated with sera prepared against emulsions heated to 58° C. for 30 minutes, with sera prepared against vibrios heated to 100° C. for 2 hours, and with a serum prepared against flagellar emulsion.

Vibrios heated to 100° C. for 2 hours agglutinated with the first two batches of sera only, but failed to agglutinate with the sera prepared against the flagellar emulsion.

It should be emphasized that the difference in the character of agglutination of living emulsions containing both the thermolabile and thermostable antigens and heated emulsions containing only the thermostable antigen is very slight ; there were no big flakes in the first case nor small granules in the second, the flakes being smaller and the granules being larger than those of typhoid and proteus examined under similar conditions. Although a difference may be noticed in one or more strains under certain conditions, yet on the whole, and after investigating the phenomenon on a large scale, the absence of marked difference under both conditions was ascertained.

This may account for the failure of earlier workers to detect the thermolabile antigen of cholera ; it would appear that the greater part of the flocculation which takes place when a cholera vibrio is acted upon by its homologous serum is due to the interaction between the thermostable antigen and its corresponding antibodies, the thermolabile antigen being only feebly represented in this reaction.

Another point of importance in connection with the agglutination of the thermostable antigen of cholera is the rate of flocculation, as in 10 to 15 minutes there was definite agglutination at the lower dilutions of the serum.

PART II.

Having ascertained the double antigenic structure of *V. choleræ*, it was thought desirable to investigate whether races of *V. choleræ* exist differing in their somatic antigens.

Living 20-hours' broth culture of all the strains of cholera, El Tor and cholera-like vibrios were put up against the two cholera antisera, prepared against vibrios heated to 58° C. for 30 minutes, and containing antibodies

corresponding to both antigens of *V. choleræ*, and against the serum prepared with the flagellar emulsion of *V. choleræ*. It was found that all the strains of cholera and four El Tor strains agglutinated almost to the same titre with all three sera, while two cholera-like vibrios agglutinated to varying titres.

Emulsions containing the thermostable antigens only of the various vibrios were prepared as follows :

Agar slopes of 20 hours' growth of all the strains of cholera, El Tor and cholera-like vibrios were emulsified separately, each in 10 c.c. of saline.

The emulsions were heated to 100° C. for 2 hours, and were then diluted to an opacity matching Brown's opacity tube No. 2 for agglutination purposes.

The diluted emulsions were put up against varying dilutions of the three anticholera sera containing antibodies corresponding to the heat-stable antigen only, being prepared against vibrios heated to 100° C. for 2 hours.

Readings were made after incubation in the water-bath at 55° C. for 3 hours, and again after being left overnight.

The result showed that all the strains of *V. choleræ* agglutinated to almost the same titre with either of the three sera used.

The absorption tests were carried out according to the technique already described. Thick suspensions of the various strains of *V. choleræ* were heated to 100° C. for 2 hours and were used as the absorbing antigens.

It was found that the heat-stable antigen of any strain of them absorbed from either of the three sera not only the agglutinins corresponding to itself, but also those corresponding to other strains of *V. choleræ* as well.

The deduction which seems to follow as a result of these findings is that the heat-stable antigen of twenty-two strains of *V. choleræ* agglutinated with and absorbed agglutinins from three sera prepared against three of them.

The four strains of El Tor vibrios, which were found to agglutinate with cholera antiserum containing antibodies corresponding to both antigens of *V. choleræ*, fell into two groups when they were put up against the three sera just mentioned and examined on similar lines. The result of the examination of both groups separately will be given later on.

All the cholera-like vibrios showed no agglutination when they were examined under similar conditions.

The Antigenic Relationship of V. choleræ *and* V. El Tor.

Neufeld and Handel (1907) and others established the serological identity of *V. choleræ* and the hæmolytic El Tor vibrios. As this work was done before the double antigenic structure of *V. choleræ* was established, it was thought desirable to investigate the identity of the thermolabile and thermostable antigens of both organisms separately.

Living cultures, cultures heated to 100° C. for 2 hours, and flagellar emulsions of six vibrios labelled " El Tor " were prepared and put up for agglutination against various sera.

Table I shows that this collection fell into three groups.

1. Group A including El Tor 1908 and El Tor 30 did not agglutinate under any condition with any of the cholera antisera ; nor did it agglutinate with a serum prepared against El Tor A.T.C.C. 582.

TABLE I.

Strains.	Serum El Tor 582.				Serum v.c.j. (H. + O.).				Serum v.c.j. (O.).			
	1/400.	1/800.	1/1600.	1/3200.	1/400.	1/800.	1/1600.	1/3200.	1/400.	1/800.	1 1600.	1/3200.
El Tor 30 (L.)	−	−	−	−	−	−	−	−	−	−	−	−
,, 30 (H.)	−	−	−	−	−	−	−	−	−	−	−	−
,, 30 (F.E.)	−	−	−	−	−	−	−	−	−	−	−	−
,, 1908 (L.)	−	−	−	−	−	−	−	−	−	−	−	−
,, 1908 (H.)	−	−	−	−	−	−	−	−	−	−	−	−
,, 1908 (F.E.)	−	−	−	−	−	−	−	−	−	−	−	−
,, 582 (L.)	+++	++	+	−	+++	++	+	−	+++	+	+	−
,, 582 (H.)	++	++	+	−	++	+	+	−	++	+	+	−
,, 582 (F.E.)	++	++	+	+	++	+	−	−	−	−	−	−
,, 47 (L.)	++	++	+	−	++	++	+	−	++	++	+	−
,, 47 (H.)	++	+	+	−	++	+	−	−	++	+	+	+
,, 47 (F.E.)	++	+	+	−	++	+	+	−	−	−	−	−
,, A (L.)	++	++	+	−	++	++	++	+	++	+	+	−
,, A (H.)	++	+	−	−	++	+	+	−	++	+	+	−
,, A (F.E.)	++	++	+	−	++	+	+	−	−	−	−	−
,, D (L.)	++	++	+	−	++	++	+	−	−	−	−	−
,, D (H.)	−	−	−	−	−	−	−	−	−	−	−	−
,, D (F.E.)	++	++	+	+	++	++	+	−	−	−	−	−

Serum El Tor 582 was prepared against emulsions of the same vibrio heated to 58° C. for 30 minutes and presumably containing antibodies corresponding to both antigens of this vibrio. Serum v.c.j. was prepared against strain Jerusalem; the one labelled (H. + O.) contains antibodies corresponding to both antigens of cholera, and the one labelled (O.) contains antibodies corresponding to the heat-stable antigen only. (L.) = Living emulsions; (H.) = Emulsions heated to 100° C. for 2 hours; (F.E.) = Flagellar emulsion.

2. Group B, including El Tor D, agglutinated in the living state with cholera antiserum, containing antibodies corresponding to both antigens, as well as with a similar serum of *V. El Tor* 582. It, however, failed to agglutinate with an anticholera serum containing antibodies corresponding to the heat-stable antigen only. Investigation of this vibrio will be given with the group of vibrios described later.

3. Group C, including the three El Tor vibrios A.T.C.C. 582, A and 47, agglutinated in the living state with the three sera mentioned. They agglutinated with and absorbed agglutinins from cholera antiserum containing antibodies corresponding to the heat-stable antigen only, and behaved as if having the same thermostable antigen as *V. choleræ*. A further consideration of Table I shows that flagellar emulsions of these vibrios agglutinated with a cholera serum as well as with an El Tor serum. By a series of cross absorptions of various sera with living and heated emulsions of *V. choleræ* and these three El Tor strains, it was established that these vibrios not only possess the same thermostable, but also the same thermolabile antigen as *V. choleræ*.

It would appear advisable to confine the term "El Tor" to the group of vibrios sharing with *V. choleræ* both its antigens, but differing in their being actively hæmolytic.

El Tor D should be included among a group next to be described, which shares with the cholera vibrio the thermolabile antigen only, the heat-stable being distinct.

El Tor 1908 and El Tor 30, which showed no antigenic relationship whatever with *V. choleræ*, may be included among the cholera-like vibrios.

Investigation of Vibrios Agglutinating with Cholera Serum in Virtue of the Thermolabile Antigen.

The result of this analysis is the detection of a group of vibrios, one labelled El Tor D, and two labelled cholera-like vibrios G.L. sewage and D 16 agglutinating with cholera antiserum, containing antibodies corresponding to both antigens, when examined in the living state, but ceasing to agglutinate on being heated to 100° C. for 2 hours.

The biological character of these vibrios resembles closely that of *V. choleræ*, except that they are actively hæmolytic to sheep's cells.

With the serum prepared against one of them, vibrio G.L. sewage, a series of agglutinations was carried out.

Table III shows that the cross agglutination between *V. choleræ* and vibrio G.L. sewage, which took place when cultures of both were used in the living state, ceased to take place when either vibrio was heated to 100° C. for 2 hours; that is to say, this agglutination is in virtue of a thermolabile antigen. Additional proof in the same direction is also furnished in Table III.

Here, although a living emulsion of vibrio G.L. sewage agglutinated with cholera antiserum containing antibodies corresponding to both antigens of *V. choleræ*, yet it failed to agglutinate with a serum prepared against the same strain of *V. choleræ*, but containing antibodies corresponding to the heat-stable antigen only.

Table II.

Strains.	Serum Jerusalem (H. + O.). 1/200.	1/400.	1/800.	1/1600.	1/3200.	Serum g.l. sewage (H. + O.). 1/200.	1/400.	1/800.	1/1600.	1/3200.
G.l. sewage (L.)	+++	+	−	−	−	+++	++	++	+	+
V.c. Jerusalem (L.)	+++	+++	++	+	+	++	+	−	−	−
G.l sewage (H.)	−	−	−	−	−	+++	++	+	+	−
V.c. Jerusalem (H.)	+++	++	++	+	−	−	−	−	−	−

Serum Jerusalem (H.+O.) is a serum prepared against *V. cholerœ* strain Jerusalem and contains antibodies corresponding to both its antigens. Serum g.l. sewage (H. + O.) is similar serum prepared against *V. cholerœ* like g.l. sewage. (L.) = Living emulsions; (H.) = Emulsions heated to 100° C. for 2 hours.

Table III.

Strains.	Serum Jerusalem (H. + O.). 1/200.	1/400.	1/800.	1/1600.	1 3200.	Serum Jerusalem (O.). 1/203.	1/400.	1/809.	1/1600.	1/3200.
G.l. sewage (L.)	++	+	−	−	−	−	−	−	−	−
V.c. Jerusalem (L.)	++	++	++	+	+	++	++	+	+	−

Serum Jerusalem (H. + O.) is prepared against *V. cholerœ* strain Jerusalem, and contains antibodies corresponding to both antigens of cholera vibrio. Serum Jerusalem (O.) contains antibodies corresponding to the heat-stable antigen of *V. cholerœ* strain Jerusalem only.

Table IV.

Flagellar emulsions.	Serum Jerusalem. 1 200.	1/400.	1/800.	1/1600.	1/3200.	Serum g.l. sewage. 1/200.	1/400.	1/800.	1/1600.	1/3200.
G.l. sewage	++	++	+	+	−	++	++	+	+	+
Jerusalem	++	++	+	+	+	++	++	+	+	+

Both sera used contain antibodies corresponding to both antigens of each vibrio.

Table V.

Strains.	Serum Jerusalem (H. + O.). 1/200.	1/400.	1/800.	1/1600.	1/3200.	Serum Jerusalem (O.). 1 200.	1/400.	1/800.	1/1600.	1/3200.	Serum g.l. sewage (H. + O.). 1 200.	1/400.	1/800.	1/1600.	1/3200.
El Tor D (L.)	−	−	+	++	++	−	−	−	−	−	−	−	+	++	++
,, D (H.	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D 16 (L.)	++	++	++	+	+	−	−	−	−	−	++	++	++	+	+
D 16 (H.)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Detached flagellar emulsions of vibrio G.L. sewage and *V. choleræ* reciprocally agglutinated with each other serum as in Table IV.

Results very closely resembling these were also obtained when the two other members of this group, D 16 and El Tor D, were examined on similar lines.

Table V shows that these vibrios agglutinated in the living state with cholera antiserum and G.L. sewage antiserum (both containing antibodies corresponding to both antigens of both vibrios); but they failed to agglutinate with either serum when they were heated to 100° C. for 2 hours.

These vibrios, as shown in Table V, failed to agglutinate in the living state as well as in the heated state with cholera antiserum containing antibodies corresponding to the heat-stable antigen only.

It is to be noticed that although vibrio G.L. sewage, previously described, agglutinated to a comparatively low titre with cholera antiserum, yet these two vibrios agglutinated to high titres with the same serum.

Further consideration of Table V shows that these vibrios failed to agglutinate with G.L. sewage antiserum after they were heated, although this serum contains antibodies to both the heat-stable and heat-labile antigens of vibrio G.L. sewage. By a series of agglutinations and agglutinin absorptions, using living and heated emulsions of these three vibrios (D 16, El Tor D, and G.L. sewage), it was established that the heat-stable antigens of this group of vibrios were not one and the same.

It is to be mentioned that this phenomenon of cross-agglutination between these vibrios and the *V. choleræ* in virtue of a heat-labile antigen is, however, irregular and inconstant. On the whole broth cultures were more suitable than saline emulsions of agar cultures for its demonstration.

DISCUSSION.

This present investigation renders the possibility of the existence of races of *V. choleræ* differing in their somatic antigen highly improbable, and establishes the generic specificity of this antigen.

The work of Landsteiner and Levine (1927) also points in the same direction. This specificity is, however, not absolute, as the classical *V. El Tor* shares with *V. choleræ* this antigen.

The International Conference, Paris (1911), recommended that a vibrio isolated from a suspected carrier agglutinating to a low titre (1 : 100), with an anticholera serum of a comparatively high titre (1 : 4000) may be regarded as a true cholera vibrio.

Non-specific agglutination with cholera antiserum of vibrios resembling the true cholera vibrio, but differing in one way or other, was reported by Sierakowski (1920), by Douglas (1921), by Mackie (1922), and more recently by Shousha (1931).

In his report on two vibrios isolated at El Tor quarantine station in 1930, Shousha concluded that the cross agglutination between them and the true *V. choleræ* was due to a thermolabile antigen.

He suggested the use of heated emulsions and sera prepared against heated emulsions for the diagnosis of *V. choleræ*.

Similar conclusions were arrived at independently before the author was aware of Shousha's work.

The epidemiological and pathogenic significance of these vibrios, which agglutinate with cholera antiserum irregularly or only to a slight extent, but differ serologically in one fraction or other of their antigen from the true *V. choleræ*, is far from being established definitely.

At least on one occasion the population of a quarantine camp was condemned "Brute," or cholera-infected because of the presence of vibrios of this nature, showing non-specific agglutination with cholera antiserum, in healthy carriers.

In an attempt to differentiate between these vibrios and the true *V. choleræ*, a serum prepared against the thermostable antigen of *V. choleræ* is to be recommended, in addition to the usual polyvalent sera containing antibodies corresponding to both antigens of *V. choleræ*, in the diagnosis of a vibrio of doubtful nature.

This first serum picks quickly and unerringly the true cholera vibrio, whether heated, formolized, or living, and fails to agglutinate with any of the cholera-like vibrios, even those sharing with the cholera vibrio its thermolabile antigen.

A vibrio agglutinating with both sera is a true *V. choleræ*; a vibrio agglutinating with the second but not with the first belongs to this group, agglutinating in virtue of its heat-labile antigen.

The position of the classical El Tor vibrios is, however, difficult, as these vibrios will agglutinate with both sera, but are actively hæmolytic.

SUMMARY.

1. The presence of a thermolabile antigen in addition to a thermostable one in *V. choleræ* is confirmed.

2. Twenty-two strains of *V. choleræ* obtained from widely different sources, all of which agglutinated in the living state with a serum prepared against the flagellar emulsion of one of them, and presumably having the same heat-labile antigen, were found to be homologous, so far as their heat-stable antigen is concerned. All agglutinated with and absorbed agglutinins from three sera prepared against the heat-stable antigens of three of them.

3. Some strains labelled El Tor, differing from *V. choleræ* in being actively hæmolytic, were found to have the same thermolabile and thermostable antigens as the true *V. choleræ*.

4. Apart from this relationship of these strains of El Tor, the heat-stable antigen of *V. choleræ* appears to be sharply specific. None of twenty-four cholera-like vibrios examined was found to share with *V. choleræ* this antigen.

5. There is a group of vibrios which agglutinated with cholera antiserum in virtue of its heat-labile antigens. The heat-stable antigens of these vibrios are entirely distinct from that of *V. choleræ*.

6. These vibrios resemble very closely *V. choleræ* in their biological characters, but they are actively hæmolytic.

7. By a series of agglutination and agglutinin absorption tests, it was found that although these vibrios may have the same thermolabile antigens, yet so far as their heat-stable antigens are concerned, they form a heterologous group.

I wish to record my grateful thanks to Prof. W. Leonard Forsyth for his kind help and useful criticism during the progress of this work.

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THE EFFECT OF STREPTOCOCCAL TOXIN ON CHINCHILLA RABBITS.

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THE titration of scarlet fever toxin and antitoxin by skin-tests on human beings has several disadvantages, one of which is the difficulty of obtaining suitable subjects. In order to overcome this, skin-tests on various animals have been used (Wadsworth, Kirkbride and Wheeler, 1926; Rosenow, 1925; Ando and Kurauchi, 1930). Skin-tests on chinchilla rabbits (Fraser and Plummer, 1930*a*) have given results comparable in accuracy to human tests. Tests on rabbits give as close agreement with human tests of the same material as can generally be obtained between two human tests of the same antitoxin. In both rabbit and human tests, however, there is often a wide variation between the readings for individual subjects in the same test. Such variations are a serious limitation to the accuracy of the test.

The protection of rabbits by antitoxin against lethal doses of living streptococci (Parish and Okell, 1927), and of streptococcal toxin (Hartley, 1928; Pulvertaft, 1928) has been carried out, and the titration of antitoxin attempted by this means.

TITRATION OF TOXIN BY LETHAL TEST.

The susceptibility of chinchilla rabbits to intravenous doses of streptococcal toxin varies to such an extent that when a dose of 1 c.c. per kg. is sufficient to kill some animals, others survive doses of 4 c.c. per kg. of the same toxin. In any attempt to titrate streptococcal toxin by a lethal test, this variation in the susceptibility of the test animals should be taken into account in estimating the accuracy of the test.

The greater the variation in susceptibility of the animals, the larger the number of animals required in order to differentiate between two doses of toxin. The variation in susceptibility can be measured by the increment in dose necessary to produce a unit increment in the mortality per cent. By injecting groups of animals with different doses of toxin and plotting the mortality per cent. against the dose injected, a curve can be obtained, the slope of which depends on the susceptibility of the test animal. The degree of accuracy to be expected from the test depends on the slope of the curve and on the number of animals used in the test.

In order to avoid possible variation from the use of more than one breed, the rabbits used were of chinchilla type, but were not pure bred, and in some cases had coats darker or lighter than the colour characteristic for chinchillas. The dose of toxin was proportional to the weight of the animal, and was given intravenously. Small doses were diluted, so that the required dose was contained in a volume of 1 c.c. per kg. of the animal's weight.

Animals weighing from 1·7 to 2·6 kg. were used, and as far as possible the average weight of each group of test animals was kept constant. Most of the deaths occurred between 12 and 18 hours after the injection of toxin ; a few occurred between 1 and 5 days, and an occasional animal died between the 6th and 11th day. The period of observation was 14 days. Autopsies were made on all animals dying within 14 days, and cultures taken from the heart, lung, liver, spleen and kidney, in order to rule out spontaneous streptococcal infections. The characteristic changes due to toxin consisted essentially of hæmorrhages ; particularly in the lung, stomach, thymus and thyroid, although hæmorrhages were usually found in other tissues as well. One animal with lung abscess was excluded from the test of precipitate X, and one with an area of pulmonary consolidation was excluded from the test of the acetone precipitation.

The toxin used was prepared by growing strain Dochez N.Y. 5 for 48 hours at 37° C. on 1 per cent. peptone beef infusion broth at pH 7·5. Similar broth containing 5 per cent peptone was used for the acetone precipitation. No difference in potency was noted between phenolized and unphenolized fractions of the same filtrate. With suitable dosage, similar results were obtained with unconcentrated toxin and preparations redissolved in normal saline or distilled water, after reduction in volume by precipitation with glacial acetic acid (Fraser and Plummer, 1930, *b*), alcohol (Ando, Kurauchi and Nishimura, 1930), or acetone (Wadsworth and Quigley, 1931). Large doses of broth and preparations of broth precipitated by acetic acid, alcohol or acetone produced no symptoms. Animals were protected against lethal doses of each type of preparation by scarlet fever antitoxin, but not by diphtheria or tetanus antitoxin.

Owing to the probable instability of the acetic acid preparations, the number of animals in each group tested was small. Redissolved dried acid precipitate was also used, but if a large quantity is dissolved at one time, this material is also subject to possible deterioration, and if small amounts are dissolved at intervals, more toxin may be left adhering to the container on one occasion than another. The number of animals used in testing dried preparations was insufficient to warrant any conclusions as to deterioration of the toxin, although it was evident from the tests that a considerable amount of toxicity was still present.

The acetone precipitate was made according to the method described by Wadsworth and Quigley (1931), except that the undialyzed first precipitate was used. Dialyzed, reprecipitated material tested on a small number of animals gave results similar to those obtained with the undialyzed precipitate.

In testing the acetone preparation, groups of 30 animals were used for each dose ; the whole group was not necessarily injected on the same day. No animal received more than one injection of toxin. The first doses injected were 0·1, 0·5 and 2·5 c.c. per kg. ; doses of 0·25, 0·5 and 1 c.c. per kg. were injected into fresh groups two weeks later, a dose of 2·0 c.c. per kg. three weeks later, doses of 0·4, 0·5 and 0·6 c.c. per kg. seven weeks later, and finally the 0·4, 0·5 and 0·6 c.c. doses were repeated three months from the time the test was begun.

It should be noted that the 0·5 c.c. per kg. dose was included in each group of doses injected The first, second, third and fourth group of rabbits injected with this dose had a mortality of 57, 37, 53 and 60 per cent. respectively. Since these variations are within the range of probable error, no deterioration of the toxin was apparent during the period of the test.

RESULT OF LETHAL TESTS.

Two preparations of acetic acid precipitate and one of acetone precipitate were tested on a sufficient number of animals to obtain mortality curves. The number of deaths and survivals for each dose is given in Table I.

The curves obtained by plotting the mortality per cent. against the doses used for each group are shown in Figs. 2 and 3. The ordinates represent the percentage of animals dying, the abscissæ represent the doses of toxin. The log of the dose is used in order to permit comparison of the slopes of the various curves. The number at each point on the curve represents the number of animals used in the group.

Similar curves for diphtheria toxin (Fig. 1) and dysentery toxin (Figs. 1, 2 and 3), taken from J. W. Trevan's paper on " The Error of Determination of Toxicity," 1927, and plotted on a similar scale, are shown for comparison. By inspection of the curves it will be seen that the slopes of the curves for streptococcal toxin are steeper than that of dysentery toxin, and, with the exception of acid precipitate II, less steep than that of diphtheria toxin. Since the number of animals used in testing the acetone precipitate is larger, more reliance is to be placed on this curve than on that of the acid precipitates. From the curves, one may conclude that the accuracy of the lethal test for streptococcal toxin is intermediate between that of the diphtheria and dysentery toxin tests.

TABLE I.

Preparation.	Dose c.c. per kg.	Number of animals.	Deaths.	Survivals.	Deaths per cent.	
Acid precipitate X	2·00	7	7	0	100	
	1·00	10	10	0	100	
	0·75	13	10	3	77	
	0·71	9	1	8	11	
	0·58	13	1	12	8	
	0·10	1	0	1	..	
Acid precipitate II.	1·00	1	1	0	..	
	0·50	41	33	8	80	
	0·25	11	6	5	55	
	0·10	7	1	6	14	
Acetone precipitate 4	2·50	30	25	5	83	
	2·00	30	25	5	83	
	1·00	30	17	13	57	
	0·60	30	15	15	50	Av. = 53
	0·60	30	17	13	57	
	0·50	30	17	13	57	
	0·50	30	11	19	37	
	0·50	30	16	14	53	Av. = 52
	0·50	30	18	13	60	
	0·40	30	6	24	20	Av. = 40
	0·40	30	18	12	60	
	0·25	30	6	24	20	
	0·10	30	6	24	20	

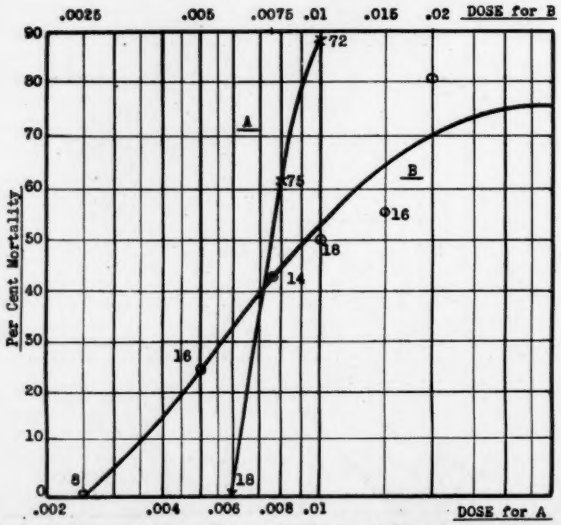

FIG. 1.—Per cent. mortality curves from Trevan (1927) for diphtheria toxin tested on guinea-pigs and dysentery toxin tested on mice. A. Diphtheria toxin. B. Dysentery toxin. The numbers attached to the observed points represent the number of animals used to determine that point.

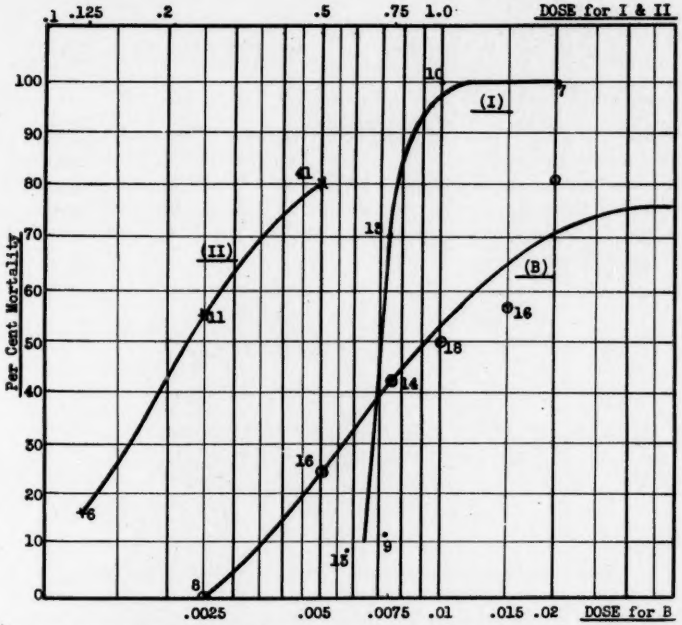

FIG. 2.—Per cent. mortality curves of dysentery toxin tested on mice (Trevan, 1927) and two preparations of streptococcal toxin tested on chinchilla rabbits. B. Dysentery toxin. I. Streptococcal toxin X prepared by precipitation with acetic acid. II. Streptococcal toxin II prepared by precipitation with acetic acid. The numbers attached to the observed points represent the number of animals used to determine that point.

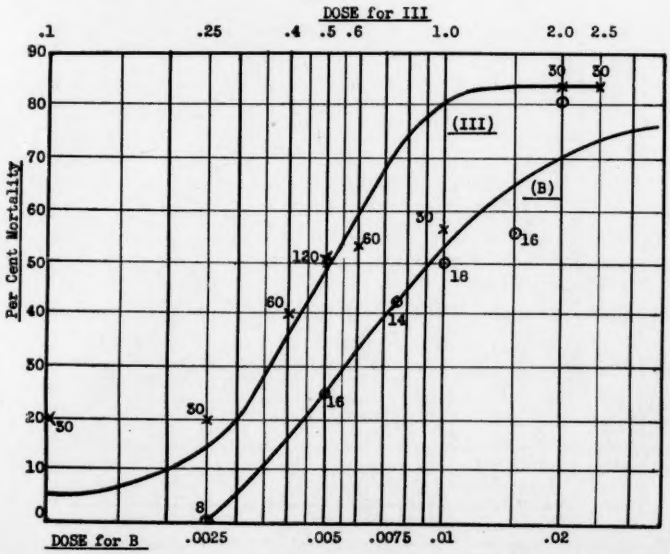

FIG. 3.—Per cent. mortality curves of dysentery toxin tested on mice (Trevan, 1927) and a preparation of streptococcal toxin tested on chinchilla rabbits. B. Dysentery toxin. III. Streptococcal toxin prepared by precipitation with acetone. The numbers attached to the observed points represent the number of animals used to determine that point.

ACCURACY OF LETHAL TESTS ON RABBITS.

According to the work of Trevan (1927, 1929, 1930), the degree of accuracy which can be obtained by lethal tests can be determined from the slope of the curve obtained by plotting the mortality per cent. against the log of the dose of toxin. The ratio R of the potencies of two toxins which will be distinguished in 95 per cent. of trials when tested on two groups of animals can be obtained from the formula—

$$\text{Log } R = 200\, A \sqrt{\frac{2pq}{N}}$$

where N = number of animals used for each test dose.
p = chance of death.
q = chance of survival.
A = the increment of the logarithm of the dose for unit increment in percentage mortality rate.

The limits of potency distinguishable from a standard of one hundred for groups of 10, 20, 30 and 50 animals obtained by this formula from each of the curves are given in Table II, and are plotted in Fig. 4.

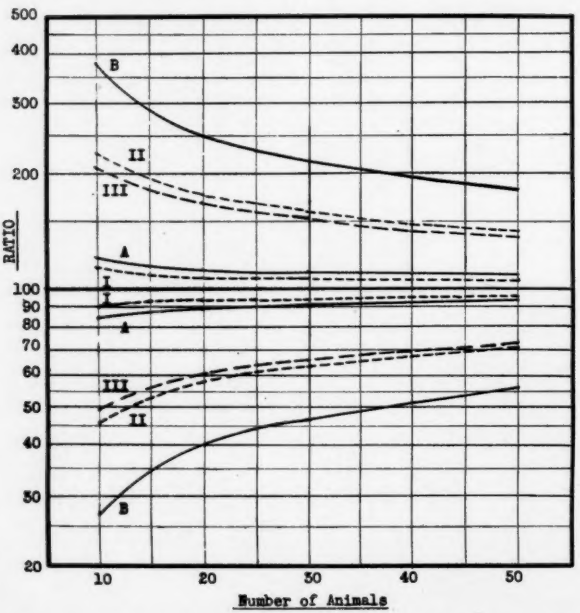

FIG. 4.—Comparison of the limits of potency distinguishable in 95 per cent. of trials by lethal tests of various toxins, showing the range of accuracy obtainable for different numbers of animals. A. Diphtheria toxin. B. Dysentery toxin. I. Streptococcal toxin X prepared by precipitation with acetic acid. II. Streptococcal toxin II prepared by precipitation with acetic acid. III. Streptococcal toxin prepared by precipitation with acetone. *Ordinates*: Ratio of potencies of standard and test where the standard equals 100. *Abscissæ*: Number of animals injected with each toxin.

TABLE II.—*The Limits of Potency Distinguishable for Different Groups of Animals.*

(Standard = 100.)

Number of animals.	Streptococcal toxin X acetic acid precipitate.		Diphtheria toxin (Trevan, 1927).		Streptococcal toxin acetone precipitate.		Streptococcal toxin II acetic acid precipitate.		Dysentery toxin (Trevan, 1927).	
	R.	Range.	R.	Range.	R.	Range.	R.	Range.	R.	Range.
10	1·11	90 to 111	1·18	85 to 118	2·03	49 to 203	2·16	46 to 216	3·65	27 to 365
20	1·07	93 „ 107	1·12	89 „ 112	1·65	60 „ 165	1·73	58 „ 173	2·50	40 „ 250
30	1·06	94 „ 106	1·10	91 „ 110	1·51	66 „ 151	1·56	64 „ 156	2·11	47 „ 211
40	1·05	95 „ 105	1·09	92 „ 109	1·43	69 „ 143	1·47	68 „ 147	1·91	52 „ 191
50	1·05	95 „ 105	1·08	93 „ 108	1·37	73 „ 137	1·41	71 „ 141	1·79	56 „ 179

TABLE III.—*Survival Time of Rabbits Injected with Streptococcal Toxin Precipitated with Acetone.*

Dose in c.c. per kg.		Day of death. 1.	2.	3.	4.	5.	6.	7.	8.	Total.	Average survival time.
0·10	Number	3	2	..	..	..	..	..	25	30	
	Survival time	3	4	..	..	..	..	..	200	207	6·9
0·25	Number	5	..	..	..	1	..	..	24	30	
	Survival time	5	..	..	..	5	..	..	192	202	6·7
0·40	Number	18	2	1	..	1	1	..	37	60	
	Survival time	18	4	3	..	5	6	..	296	332	5·53
0·50	Number	47	6	1	2	3	..	2	59	120	
	Survival time	47	12	3	8	15	..	14	472	571	4·76
0·60	Number	28	4	..	..	..	..	..	28	60	
	Survival time	28	8	..	..	..	..	..	224	260	4·33
1·00	Number	13	3	..	..	..	..	1	13	30	
	Survival time	13	6	..	..	..	..	7	104	130	4·33
2·00	Number	21	..	2	1	..	1	..	5	30	
	Survival time	21	..	6	4	..	6	..	40	77	2·57
2·50	Number	16	3	2	2	2	..	..	5	30	
	Survival time	16	6	6	8	10	..	..	40	86	2·87

SURVIVAL TIME.

Although most of the animals dying after the injection of toxin did not survive more than 48 hours, a small proportion died after several days. That the relative number dying later than 48 hours after injection was no greater for the small doses than for the larger doses of toxin may be seen from Table III. By plotting the average survival time for each dose against the logarithm of the dose, no improvement over the slope of the curve for mortality per cent. (Fig. 4) was obtained. In determining the average survival time, a time limit of 8 days was taken, since only two animals died between the 8th and 14th days, and the shorter time limit reduces the standard deviation of the individual observations.

The increased accuracy obtainable in the case of diphtheria and dysentery toxins by the use of a multiple of the average lethal dose of toxin for the titration of antitoxin (Trevan, 1927, 1930; Blake and Okell, 1930), suggests that in the titration of streptococcal antitoxin against a multiple dose of toxin, a greater degree of accuracy might be expected than in the titration of toxin alone.

SUMMARY.

1. The variation in the individual susceptibility of chinchilla rabbits to intravenous doses of streptococcal toxin can be determined from the slope of a curve obtained by plotting the mortality per cent. against the corresponding dose of toxin.

2. Per cent. mortality curves are given for three preparations of streptococcal toxin. From the slope of these curves the accuracy of titrations of streptococcal toxins by a lethal test on rabbits is determined.

3. The accuracy obtainable by the method described is intermediate between that of lethal titrations of diphtheria and of dysentery toxin.

4. In 95 per cent. of the trials the accuracy of the test permits the differentiation of two toxins by the use of a group of 30 animals for each, provided the difference between their potencies is 33 per cent. of the potency of the stronger toxin.

5. Within the limits of accuracy of the test, no deterioration was noted in toxin precipitated by acetone after storage for three months at 9° C.

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THE PRODUCTION OF COCCAL FORMS OF *C. DIPHTHERIÆ* IN MEDIA CONTAINING COPPER.

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COCCOID forms of *C. diphtheriæ* have been described by several workers. Heinemann (1917) found coccal forms present in certain broth media prepared from beef, veal or horse muscle. The toxin produced in these cases was of low potency, but otherwise seemed similar to that produced by the bacillary form. Yarisawa (1926) found that coccal forms appeared when cultures of freshly isolated *C. diphtheriæ* were grown on blood-agar, but the coccal forms reverted to the bacillary type when injected into experimental animals. He attributed the change to the coccal form to the use of insufficiently heated serum medium, and produced this change with some strains by using insufficiently heated Loeffler's medium. When grown in ordinary broth his strains showed only the bacillary form. Parish (1927) has reported the production of coccal forms in medium prepared by the tryptic digestion of muscle, according to the method of Watson and Langstaff (1927). In this case the coccal forms were observed when the broth was used for routine production of diphtheria toxin, the strain being the customary Park 8. When transferred from this broth to agar, prepared from tryptic broth, the organism grew in the coccal form as it did also on Hiss's serum water, Witte peptone water, serum and glucose broths and blood-agar. Subcultures on these media were not carried beyond the first or second generation. On the other hand, transfer to Loeffler's medium led to rapid reversion to the bacillary form. From his results Parish concluded that once the organism had assumed the coccal form, it tended to retain that form unless placed on an eminently favourable medium, such as Loeffler's blood-serum. His attempts to obtain the coccal form on other than tryptic digest medium failed. Although he obtained various changes in morphology, he was unable to produce the typical coccal forms, obtained with the special batch of tryptic digest medium, at will.

The observations to be reported in this paper arose out of experiments carried out to determine the influence of additions of copper on the production of toxin by *C. diphtheriæ*. During these experiments it was found that at certain concentrations of copper there was a change in the form of the organism from the bacillary to the coccal form.

The medium used throughout this work was prepared by the tryptic digestion of ox heart muscle, and is based on the tryptic digest type of media worked out by Douglas (1914) and Hartley (1922). It is particularly suitable for the routine production of high value toxin, and differs from that of Watson and Langstaff in that the digestion is carried out at a temperature of 50° C.

instead of at room temperature for 3 days. During some four years' experience with the routine production of toxin on both tryptic digest and infusion peptone broths we have never encountered a broth giving the coccal forms described by Parish. It would appear, therefore, that the change from bacillary to coccal forms on broth medium is exceptional, and that when it does occur, it is probably due to the presence of an abnormal constituent in the medium.

EXPERIMENTAL.

A batch of ox muscle digest broth was adjusted to pH 8·0 and divided into six portions. Copper in the form of copper sulphate was added to give concentrations of 0·025 to 50·0 mgm. Cu ion per litre of broth, one portion being retained as control. Each portion was sterilized by filtration through Berkefeld candles, the medium being distributed in 200 c.c. quantities in litre-capacity bottles. These, after incubation for sterility, were inoculated from a young Loeffler slope culture of *C. diphtheriæ* (Park 8 strain). After 5 days' incubation the cultures were examined microscopically. It was then found that where the medium had contained 50·0 mgm. Cu per litre the organisms were all coccal in form, whereas in the medium containing the smaller amounts of copper the organism presented the normal bacillary appearance. On transfer to Loeffler's medium, the coccal forms at once reverted to the bacillary form.

It is not proposed in this paper to consider in detail the influence of copper on toxin production, but it is of interest that, while in the control broth and the samples to which copper up to 5 mgm. was added, the Lf. value (Glenny and Okell, 1924) ranged from 17–24 in 24 experiments, in the culture containing 50 mgm. no detectable toxin was produced. In a second experiment the same batch of ox muscle digest broth was used, and the copper concentrations were from 10 to 50·0 mgm. per litre. The details of sterilization, volume of broth used and size of bottles are exactly as described for the first experiment, and the strain was also Park 8. In the control broth and at 10 mgm. copper per litre the bacillary form was present; above this level only coccal forms (see Table I).

TABLE I.—*Showing the Results obtained with Tryptic Digest Broth containing Copper.*

		Mgm. of copper per litre.				
	Control.	10·0	20·0	30·0	40·0	50·0
Lf. units per c.c.	15*	14	1	1	0	0
(6 experiments in each group)	(13–17)*	(12–14)	(0–1)	(0–1)	..	..
Form	Bacillary	Bacillary	Coccal	Coccal	Coccal	Coccal

Again there was a marked drop in the toxin production when the organism assumed the coccal form. Smears made at various periods during growth

* 15 = average; 13 = minimum; 17 = maximum.

showed that the organism assumed the coccal form from the start, and remained unchanged during the 5 days' incubation.

It was noticed that the medium had a blue colour with 10 mgm. or more of copper, but this disappeared after about 4 days in the incubator, and was replaced by a greyish colour. The added copper appeared to be slowly combining with some material present in the broth.

The next experiment was carried out with material sterilized by filtration through a Seitz filter followed by 10 minutes' steaming at 100° C. Unfortunately there was no more of the broth previously used, and the experiment was therefore carried out with a second batch of broth prepared in a similar manner. In this experiment the concentrations of copper added were from *nil* to 25·0 mgm. per litre. After steaming at 100° C. the blue colour had disappeared except at 25 mgm. per litre, where a little remained. From the experience gained in the previous work it was decided to extend this experiment to cover several generations, and those cultures showing the coccal form were sown on to control broth ; also on to broth containing the same and a higher concentration of copper. Using broth which had been heated with copper, it was found that even at the highest concentration used, the organisms did not change entirely to the coccal form. They were always mixed with a very short bacillary form. In the first generation the mixed coccal and short bacillary forms were obtained at a copper concentration of 15·0 mgm. per litre. At lower concentrations only the bacillary form was found. In addition, the growth was considerably slowed at concentrations of 15·0 mgm. or more. When transferred to control broth, the coccal form was rapidly replaced by the bacillary form, and the second and subsequent generations on broth containing 15·0 mgm. of copper lost their coccal form and became bacillary. However, all transfers up to the fourth generation remained coccal in broth containing 20 mgm. copper per litre. On broth containing 15 mgm. of copper the second and later generations showed little signs of delayed growth, so that delayed growth would appear to be associated with the coccal form, particularly as the 20 mgm. broth maintained both the coccal form and showed delayed or slow growth. The results of this experiment are set out below in Table II:

TABLE II.

	Mgm. of Cu per litre added.				
	Nil.	10·0	15·0	20·0	25·0
Lf. value	18*	13	15	12	6
(6 experiments in each group)	(14–24)*	(10–17)	(13–17)	(9–18)	(0–13)
Microscopically	Bacillary	Bacillary	Coccal and short bacillary	Coccal and short bacillary	Coccal and short bacillary

Table III shows typical results extending to the fourth generation for one of the series which showed the coccal form in the first generation.

In each case the 20 mgm. culture showing the mixed coccal and bacillary forms was re-sown on to control, 15 mgm. and 20 mgm. medium. The figures given in brackets refer to the number of Lf. units per c.c. It will be seen

* As previous table.

TABLE III.—*Showing the Results obtained up to the Fourth Generation with Varying Amounts of Copper in Tryptic Digest Broth Sterilized by Heat.*

(1st generation) 20 mgm. Cu culture mixed forms (8) Lf. per c.c.

	Control.	15 mgm.	20 mgm.
(2nd generation)	Bacillary (11)	Bacillary (11)	Chiefly coccal (8)
(3rd generation)	Bacillary (14)	Bacillary (13)	Chiefly coccal (8)
(4th generation)	Bacillary (15)	Bacillary (7)	Chiefly coccal (3)

that after the first generation the 15 mgm. broth failed to maintain the organisms in the coccal condition, while the 20 mgm. medium did so regularly.

In this medium, which had been heated with copper, the results for toxin production as related to morphology are obscured by the fact that even at the highest concentration, some very short bacillary forms were always present. Nevertheless the strength of toxin produced diminished in the higher concentrations of copper while the proportions of coccal forms increased. It is of interest to find that when cultures consisting almost entirely of the coccal form are resown on to control medium, the toxin production is unaffected. Even as high a concentration as 15 mgm. copper per litre had little effect on toxin production until the third generation, but continual growth in media containing 20 mgm. diminished toxin production.

One further experiment calls for brief mention. In this we added, in addition to copper, iron in the form of ferric citrate (from 50 to 200 mgm. per litre). The amount of iron added was ten times that of copper in each case. No culture grown on these mixtures showed the coccal form, although in some cases extremely short bacillary forms were produced.

DISCUSSION.

It has been shown that by growing *C. diphtheriæ* in a tryptic digest medium containing copper the organism can be changed from the bacillary to the coccal form. The change occurred when the medium contained between 10 and 20 mgm. of copper ion per litre, but while it was complete in medium sterilized by filtration, it was never complete in media sterilized by heat. Two points may be considered in reference to this difference of behaviour depending on the method used for sterilization. First there is the rapid disappearance of the blue colour, produced on addition of those concentrations of copper which cause the change in morphology, when the medium is heated—as compared with its slow disappearance at room temperature or 37° C. in unheated medium. We would suggest that ionic copper is responsible for the change in form, and that combined copper is either inactive or has much

less activity. Unfortunately at the time these experiments were carried out we did not determine the amount of free copper present before and after heating, and we are therefore unable to say to what extent the amount of ionic copper was changed during heat sterilization.

The second point of interest is that in medium containing copper in amounts too low to cause the change of form, the growth was typical of *C. diphtheriæ*, a complete pellicle occurring at the surface of the medium, whereas when the coccal form occurred, the growth was at the bottom of the liquid. In the experiments with heated medium the change of form was never complete; the difference in manner of growth was also less clear cut.

We have not studied the influence of copper in medium produced by methods other than the tryptic digestion of ox heart muscle, so that we cannot be sure that the change we have described will always occur in medium containing copper in suitable concentration. Allison and Ayling (1929) have described a medium suitable for the isolation of *C. diphtheriæ* which contains copper, added as copper sulphate for the purpose of inhibiting staphylococcus, streptococcus, and *B. proteus*. Allison (1930) suggests from 0·01 per cent. to 0·05 per cent. of copper sulphate as a suitable concentration for their medium, as this range allows growth of *C. diphtheriæ*, and at the same time inhibits growth of other organisms. This amount corresponds to a range of from 25–125 mgm. of copper ion per litre. Allison and Ayling do not mention the appearance of a coccal form of *C. diphtheriæ* on their medium, so that it is highly probable that the constitution of the medium modifies the effect of copper.

The difference may be because their medium contains about 10 per cent. of horse-serum which might combine with a large amount of the copper, or because with their medium (which is solid) growth occurs on the surface, whereas in broth medium we found that the coccal form grew at the bottom. It is possible that some of the difficulty which has been found in preparing Allison's and Ayling's medium at this and other laboratories may be explained on the basis of the amount of free or combined copper present in the finished medium.

We cannot say definitely whether the coccal form of *C. diphtheriæ* produces toxin. The culture filtrates were tested by the flocculation method, and where a value of 0 Lf. units per c.c. was obtained, they were not further tested by more sensitive animal methods for traces of toxin. Thus it would be quite possible for those filtrates shown as containing 0 Lf. units per c.c. to contain some minute quantity of toxin. It is, however, obvious that the toxigenic power of the bacillary is much higher than that of the coccal form in the concentration of copper used.

Cultures grown on media containing 20 mgm. of copper and consisting very largely of the coccal form, even after three generations on the copper medium, appeared to regain at once their toxigenic power when re-sown on to control media containing no copper. Although these cultures were not entirely coccal, we have shown that pure coccal cultures rapidly revert to the bacillary form when sown on to control media, so that it seems clear that copper may produce a marked change in the morphological appearance, without having much effect on the subsequent power of the organism to produce toxin when transferred to suitable medium.

SUMMARY.

Growth of *C. diphtheriæ* on digest medium containing 20 mgm. copper per litre gave a wholly coccal form of organism provided that sterilization had been carried out by filtration through Berkefeld candles. Where the medium had been sterilized by heat, the growth consisted of mixed short bacillary and coccal forms. The coccal forms produce little or no toxin.

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THE NATURE OF THE ELEMENTARY BODIES IN PSITTACOSIS.

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IT has been recognized for some time now that it is possible in the case of many filtrable viruses to demonstrate the presence of minute bodies in virulent material, the so-called elementary bodies, of which the best-known examples are the Borrel bodies of fowl-pox and the Paschen bodies of vaccinia. Yet, despite the fact that these bodies have been known of for so long, it is still a matter of dispute as to whether they are the virus; in fact, until comparatively recently the majority of virus workers were prepared to dismiss them as something non-specific, the result of cell breakdown, or even the precipitation of protein or stain in the preparations. Latterly, however, the point of view which identifies these minute bodies with the virus has received considerable support. The beautiful experiments of Woodruff and Goodpasture (1929, 1930) on the infectivity of the isolated Bollinger body indicate very strongly that the Borrel bodies—the minute bodies which occur inside the Bollinger

body—are the virus of fowl-pox, and the recently published experiments of Ledingham (1931) on the specific flocculation of the Borrel and Paschen bodies is even weightier evidence of their ætiological importance.

ELEMENTARY BODIES IN PSITTACOSIS.

General Description.

The demonstration that the ætiological agent of psittacosis belonged to the filtrable viruses, was followed quickly by the announcement that minute bodies could be seen in suitably stained preparations of virulent material. This observation was made independently by Levinthal (1930), Lillie (1930) and Coles (1930), and since that time the majority of those who have worked with the virus of psittacosis have seen and studied these elementary bodies. They are small round or slightly oval bodies resembling minute cocci, which in smear preparations occur singly, in pairs or in irregular clumps (Fig. 1), the last arrangement being due to their intracellular habit of growth. Bacillary forms have been described (Coles, 1930; Lillie, 1930), and in earlier work (Bedson and Western, 1930) this was thought to be correct. Subsequently, however, confirmation of this has not been obtained, and from an examination of a large amount of material of mouse, guinea-pig and parrot origin, the opinion now formed is that these bodies are typically round or slightly oval, closely resembling, though somewhat larger than, the Paschen bodies. After staining for 24 hours in dilute Giemsa (1 drop per c.c.) and differentiation in an orange G tannin solution, they take on a purple colour and stand out well, but they can also be stained by means of well ripened Löffler's methylene-blue, dilute carbol fuchsin (prolonged staining), or any of the polychrome blues, such as Borrel blue. Perhaps for rapid staining nothing is more satisfactory than Rivers' modification of Castaneda's rickettsia stain. This modification has the following composition :

Phosphate buffer pH 7·0 . .	95 c.c.
Formalin	5 ,,
Löffler's methylene-blue . . .	10 ,,

Smears are stained for 2–5 minutes, washed and counter-stained for 10 seconds with a 1 per cent. aqueous safranin solution. In such preparations the bodies stain a bright blue while the cells and background appear pink. Characteristically in sections and imprint preparations made from organs, these minute bodies appear inside the reticulo-endothelial cells (Figs. 2 and 3), which are sometimes filled to bursting-point. In smear preparations, of course, with the consequent disruption of the cells, many extracellular forms are to be found.

Relation of the Elementary Bodies to the Virus.

The question naturally arises whether or not these minute bodies are the virus, and the experiments recorded below have been carried out with the object of answering this question. In favour of these bodies being the virus are the organism-like appearance which they present, their occurrence in virulent material from different sources (man, parrot, mouse, guinea-pig—see

Figs. 1, 2 and 3), and the fact that filter and centrifuge experiments would lead one to expect the virus to be represented by particles about the size of these minute bodies (Bedson and Western, 1930). Against this view, at any rate at first sight, is the fact that it is difficult, if not impossible, at times to demonstrate these bodies in virulent material (Bedson and Western, 1930; Rivers and Berry, 1931; Elkeles and Barros, 1931). This difficulty is met with chiefly in material from human and avian sources, for in preparations made from the spleens of infected mice and guinea-pigs the author has been able to demonstrate these bodies with regularity, and in earlier work (Bedson and Western, 1930) the opinion was expressed that in the case of mouse material (spleen) the number of bodies ran more or less parallel with the virulence. Subsequent work has not led to any appreciable modification of this opinion, though the findings of Rivers and Berry (1931) are not altogether in keeping with this view. However, the inability to demonstrate these bodies in virulent material with regularity is no sound reason for thinking that they are not the virus; parallel examples in the case of other organisms can readily be found.

Concentration of Psittacosis Virus by Means of the Centrifuge.

Experiments have been placed on record (Bedson and Western, 1930), showing that psittacosis virus could be thrown down appreciably by centrifugation at a speed of 4000–5000 r.p.m. Further experiments have demonstrated this even more conclusively. In two experiments suspensions of virulent mouse spleen, after clarification by a preliminary centrifugation for 10 minutes at 2000 r.p.m., were centrifuged for 2 hours at 5000 r.p.m. The supernatant fluid was then pipetted off as completely as possible, and the deposit taken up in a volume of saline diluent equivalent to the original volume. The virus content of the uncentrifuged suspension and the suspension of centrifuged deposit was then determined by intradermal titration in the guinea-pig's skin. The results are given in Table I, and show how small an amount of virus is lost in such an operation.

TABLE I.—*Centrifugation of Psittacosis Virus.*

Virus suspension.	Titration of virus. Before centrifugation. 10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	Suspension of deposit. 10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.
"Bangor." Spleens M.303/4 suspended in 10·0 c.c. Tyrode	+	±	−	−	±	∓	−	−
"Bangor." Spleen M.351 suspended in 5·0 c.c. M/50 phosphate pH 7·6 . .	+	±	∓	−	+	±	∓	−

In the protocols ++++, +++, ...∓ represent diminishing degrees of the reactions under consideration; −, a negative reaction; 0, no test.

In a third experiment 6·0 c.c. of a suspension of virulent mouse spleen in phosphate buffer (strain "Bangor"), which had been clarified by a preliminary centrifugation at 2000 r.p.m., was centrifuged for 2 hours at 5000 r.p.m. The

supernatant fluid was pipetted off completely and kept (1st supernatant). The deposit was suspended in 3·0 c.c. phosphate buffer, lightly centrifuged, the supernatant pipetted off and centrifuged for 1½ hours at 5000 r.p.m. The supernatant was then pipetted off completely (2nd supernatant), and the deposit taken up in 3·0 c.c. phosphate buffer. The virus content of the uncentrifuged suspension, the 1st and 2nd supernatants and the suspension of washed deposit was then determined by titration in the guinea-pig's skin, with the following result :

	Dilutions.				
	Undiluted.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.
Before centrifugation .	0	++	+	±	—
Suspension of deposit .	0	+	+	?	—
1st supernatant . .	±	—	—	—	—
2nd supernatant .	∓	—	—	—	—

Here, again, there has been remarkably little loss of virus, suggesting, as do the previous experiments, that psittacosis virus is represented by particles of a considerable size. In all these experiments stained preparations made from the uncentrifuged suspensions, and the deposits obtained after centrifugation, left little doubt that the elementary bodies were very appreciably concentrated by centrifugal force.

Purification of Psittacosis Virus by Fractional Centrifugation.

The foregoing experiments suggested the possibility of purifying the virus, and the following experiments were carried out with this object. In all these experiments the procedure was the same. A suspension of virulent mouse spleen, which had undergone a preliminary clarification, was centrifuged for 2 hours at an approximate speed of 5000 r.p.m. The supernatant fluid was then pipetted off completely, and the deposit taken up in saline. This suspension was then centrifuged for 10 minutes at 2000 r.p.m., the supernatant pipetted off and centrifuged for 2 hours at 5000 r.p.m. The deposit so obtained was resuspended in saline, and submitted again to the fractional centrifugation, resuspended in saline and finally lightly centrifuged to throw down any coarse particles. The supernatant fluid from this final centrifugation represented a suspension of twice-washed virus. The virus content of the uncentrifuged suspensions and suspensions of twice-washed deposit was determined by titration in the guinea-pig's skin, and the protein content by adding 1 part of a saturated solution of salicyl-sulphonic acid to 9 parts of progressive dilutions of the suspensions and noting the degree of precipitation or opacity produced.

Fig. 1.—Mag. × 2400. Stain : Giemsa. Orange G-tannin. Smear of virulent mouse spleen. Shows numerous elementary bodies lying free.

Fig. 2.—Mag. × 2000. Stain : Giemsa. Orange G-tannin. Imprint preparation of virulent guinea-pig spleen. Shows numerous elementary bodies inside a mononuclear cell.

Fig. 3.—Mag. × 2000. Stain : Eosin orange and toluidine blue. Section of liver of parrot with psittacosis. Shows elementary bodies inside a Kupfer cell.

Fig. 4.—Mag. × 2400. Stain : Carbol fuchsin after mordanting with formalin-acetic acid-tannin. Smear of twice-washed deposit M464/5. Shows that the particulate matter of twice-washed virus consists of elementary bodies.

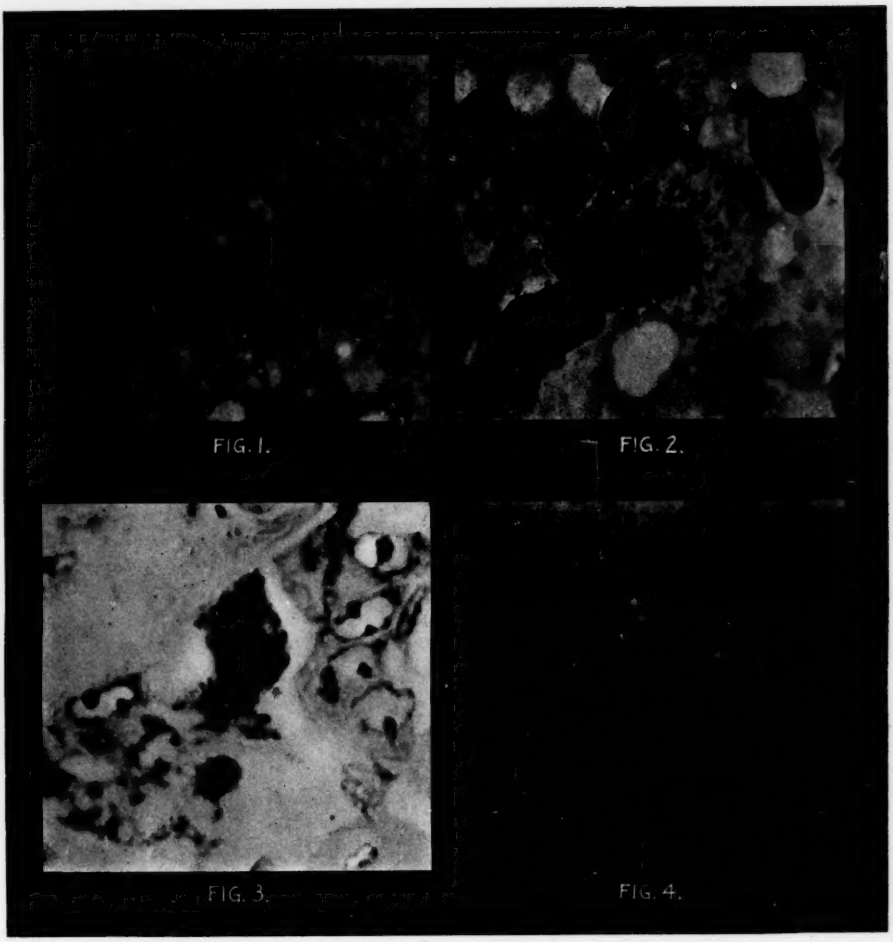

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

In all three experiments a parrot strain of virus P.XV was used, and the only way in which the three experiments differed from one another was that in experiment M. 488/9 the twice-washed deposit was suspended in a volume of saline equivalent to that of the original virus suspension, whereas in the other two only half that amount was used. The results of the virus and protein titrations are given in Table II.

TABLE II.—*Purification of Psittacosis Virus by Fractional Centrifugation and Washing.*

Experiment.		Titration.	Dilutions: Undiluted.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.
M. 448/9	Virus	Uncentrifuged	+	±	∓	—	—
		Washed deposit	+	±	—	—	—
	Protein	Uncentrifuged	++	+	±	?	—
		Washed deposit	?	—	—	—	—
M. 464/5	Virus	Uncentrifuged	++	+	+	±	0
		Washed deposit	+	+	±	∓	0
	Protein	Uncentrifuged	++	+	±	?	—
		Washed deposit	?	—	—	—	—
M. 482/5	Virus	Uncentrifuged	0	+	±	∓	—
		Washed deposit	0	+	±	?	—
	Protein	Uncentrifuged	+++	+	∓	—	—
		Washed deposit	±	—	—	—	—

These experiments give further proof that one is able to spin out the virus, and show that by means of washing and fractional centrifugation the virus can be freed from extraneous protein. In every case stained smears of the twice-washed deposit were examined, and these showed that, apart from occasional bits of extraneous material which are almost unavoidable, the only particulate material in the deposit was the minute bodies similar in every respect to those seen in smears and sections made from virulent material. Fig. 4 is a photomicrograph of such a smear made from the twice-washed deposit in Exp. M. 464/5, and shows clearly the nature of the deposit. It follows from these experiments that the virus of psittacosis is either adsorbed on these particles, or that these particles are the virus.

SEROLOGICAL EXPERIMENTS WITH PURIFIED PSITTACOSIS VIRUS.

The principal difficulty encountered in this part of the work has been the obtaining of a satisfactory antipsittacosis serum. In earlier neutralization experiments, the serum of mice which had been immunized with formolized virus and had survived a dose of living virus was used. Subsequent batches of mice treated in the same way did not furnish so good a serum as the first, and for obvious reasons the mouse is not a satisfactory animal for serum production. It was attempted, therefore, to produce antisera in the guinea-pig. This animal is not nearly so susceptible to psittacosis as the mouse, and if

given even a large dose of virus intraperitoneally, shows merely a rise of temperature up to 105–106° F., coming on in 24–48 hours and lasting two or three days. There are no constitutional symptoms, and the animal becomes refractory to subsequent inoculations. By repeated inoculation, with guinea-pig virus, of course, it has been found possible to produce sera in the guinea-pig which agglutinate psittacosis virus and fix complement in its presence. They were by no means potent sera, and only acted in low dilution, but they were sufficiently good for the purpose. Incidentally the neutralizing power of these sera, though definite, was of a low order. Since it was possible that the minute bodies were really tissue particles on which the virus was adsorbed, and since the virus used in these experiments came from mouse spleen, a serum against mouse spleen was prepared in the guinea-pig in order to find out if these washed bodies possessed a mouse spleen factor.

Agglutination Experiments.

These were carried out by the microscopic method in hanging-drop preparations. Five sera were employed :

P. 105 and P. 112. Anti-psittacosis sera prepared in the guinea-pig with guinea-pig virus.

P. 123/4. An anti-mouse spleen serum prepared in the guinea-pig.

S. 4 and S. 8. Anti-herpes sera, prepared in the guinea-pig.

Two antigens were used :

1. A suspension of twice-washed psittacosis virus prepared from virulent mouse spleen.
2. A suspension of normal mouse spleen.

The preparations were placed in the 37° C. incubator for 24 hours, after which the readings were made. The result of such an experiment is given in Table III.

TABLE III.—*Specific Agglutination of Washed Elementary Bodies.*

Sera.		Antigens. Washed psittacosis virus.	Antigens. Normal mouse spleen.
P. 105	1/1	+	−
	1/2	+	0
	1/4	±	0
P. 112	1/1	++	−
	1/2	+	0
	1/4	+	0
P. 123/4	1/1	−	+++
	1/2	0	+++
	1/4	0	++
	1/8	0	+
S. 4	1/1	−	0
S. 8	1/10	−	0

It is quite clear that the washed virus particles are agglutinated by both the antipsittacosis sera P. 105 and P. 112, but they remain unaffected by the antispleen serum which flocculates normal mouse spleen, and, of course, they are not affected by either of the antiherpes sera.

Complement-fixation.

The technique of the test was that described by Bedson and Bland (1929). In the case of all three antigens, double the amount used in the actual test did not inhibit the action of 1 M.H.D. of complement. The anti-spleen serum P. 123/4 when titrated in the presence of mouse spleen gave complete fixation in a dilution of 1 in 40; it was used diluted 1 in 10 in the test. The anti-psittacosis serum P. 112 had only a low complement-fixing titre, giving a 4+ reaction when undiluted and a 1+ reaction diluted 1 in 8; it was used diluted 1 in 2 in the test. The result of an experiment is given in Table IV.

TABLE IV.—*Complement-fixation with Washed Elementary Bodies.*

Sera.	Antigen + 2 M.H.D. complement.			Saline + 2 M.H.D. complement.
	Suspension of virulent mouse spleen.	Suspension of twice-washed psittacosis virus.	Suspension of normal mouse spleen.	
P. 112. 1/2 .	++++	+++	— .	—
	++	+	— .	—
P. 123/4. 1/10 .	++++	—	++++ .	—
	++++	—	+++ .	—
Saline . . .	—	—	— .	—
	—	—	— .	—

The upper reading is after half an hour at 37° C.; the lower after standing overnight at room temperature.

The result leaves no doubt that the washed bodies possessed no mouse factor in their make-up, and although they did not fix complement so well with the specific serum as did the virulent mouse spleen suspension, the reaction would appear to be specific, and is additional evidence of the specific nature of the elementary bodies.

CONCLUSIONS.

1. Psittacosis virus can be thrown down almost completely by centrifugation at a speed of about 5000 r.p.m.

2. The virus can be freed from extraneous matter by fractional centrifugation and washing.

3. The only particulate matter in a twice-washed deposit consists of minute bodies similar in every respect to those seen in preparations made from virulent material.

4. The washed bodies are agglutinated specifically by an anti-psittacosis serum and fix complement in its presence, but do not react in either way with an anti-mouse spleen serum.

5. These results warrant the conclusion that the minute bodies are the virus.

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THE NON-APPLICABILITY OF THE SCHÜTZ-BORISSOW LAW IN THE ESTIMATION OF PEPSIN ACTIVITY BY METT'S METHOD.

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WHILE engaged upon the study of gastric secretion following certain operative procedures on the human subject, such as partial gastrectomy, gastro-enterostomy, etc., we wished to extend our investigations so as to include variations in pepsin concentration. For our purposes we needed a method of a fair degree of accuracy and one not too laborious, because a large number of samples had to be estimated simultaneously. The method which seemed to answer our purpose best was the one devised by Mett. Although we feared that its accuracy might not compare well with the more elaborate methods, yet its extreme simplicity amply compensated for any disadvantage in other directions. Having employed it extensively, we now record some general observations made in the course of our work.

In the technique adopted the egg-albumen capillary tubes were constructed according to Christiansen's method (1912). Duplicate tests were found to correspond closely. The bore of the capillary tubes did not affect the results appreciably, though it was found that a bore of $1\frac{1}{2}$ mm. was most effective in giving a sharp line of demarcation between the solid and liquid phase, and thus facilitated the reading, which was made with the aid of a pocket lens to within $\frac{1}{4}$ mm. Both ends were read and the mean was taken, and in most instances both ends gave practically the same reading.

Where the pepsin concentration was high, however, it was observed that

the readings at the two ends were not always in agreement. It was therefore felt that the method might not be suitable for higher concentrations of pepsin. In consequence it was decided to estimate samples, in which the concentration was above a certain limit, a second time, employing a dilution higher than usual, and calculating the pepsin concentration in the given sample from the reading obtained at the greater dilution.

The concentration of pepsin was calculated on the basis of the Schütz-Borissow formula, *i. e.* the concentrations were taken as proportional to the square of the length digested ($p \propto l^2$).

A series of preliminary experiments were performed to see how close was the correspondence among results calculated on this basis from different dilutions. It was found that the figures for pepsin concentration obtained from the greater dilutions were always higher than those obtained from the lesser. Inspection revealed that for a given peptic fluid, nearly concordant values could be obtained if readings, in terms of length digested, were cubed instead of squared. To illustrate this point a series of readings are recorded in Table I.

TABLE I.—*Relation between Length Digested and Concentration of Pepsin.*

Dilution.	Length digested in ½ mm.	Length^2.	Length^3.
1 : 8	13	169	2197
1 : 16	10	100	1000
1 : 32	8	64	512
1 : 64	6	36	216
1 : 128	5	25	125

These figures clearly show that the results agree far more closely with the cube rule than with the square rule.

The accompanying graph summarizes our findings. The actual readings are compiled from graded dilutions of a series of fluids standardized by the formula 100 units = 10 mm./2 in 24 hours. On the same basis the square root law is represented by the relationship $p = l^2$, or $\log p = 2 \log l$, and the cube root law by the expression $p = l^3/10$, or $\log p = 3 \log p - 1$; and when these relationships are recorded along with the experimental determinations, as has been done on the chart, it is clear that the latter show considerable deviation from the square root law and follow quite closely the cube root law.

Commercial preparations of pepsin from different sources diluted to give approximately the same range of pepsin concentration as fractional test-meal samples yielded exactly similar results.

It was thought necessary to examine other possible factors which might be responsible for the observed discrepancy. Defective circulation in the capillary tubes would lead to a diminution of the digestion within the tube for two reasons. Firstly the process of digestion would diminish the quantity of pepsin in contact with the solid phase, and secondly, the accumulation of digestive products would have a buffering action and also a back reaction. However, experiments showed that defective circulation was not an important factor. Capillary tubes placed in fluid highly coloured with dyes, such as

methylene-blue, demonstrated a very good circulation. Further corroborative evidence was obtained as follows :

Mett's tubes, about twice the usual length, were left in a pepsin solution for several days, readings being taken 24-hourly. The column of albumen digested as shown by fresh control tubes introduced every 24 hours was the same each day, showing that the pepsin solution did not vary in strength. The additional length digested each day in the long tubes remained constant, and did not diminish as the surface of contact between liquid and solid phase

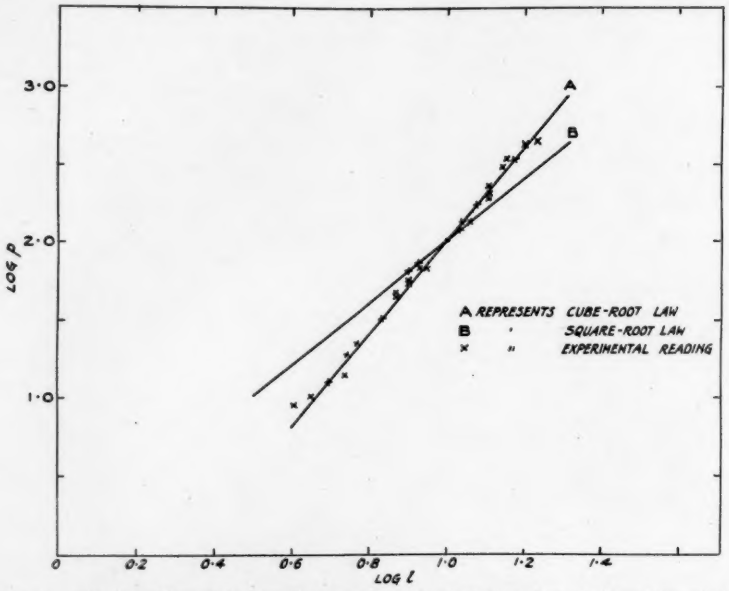

FIG. 1.—The logarithm of the pepsin concentration plotted against the logarithm of the length of the column of albumen digested, measured in half millimetres.

moved further away from the end of the capillary tube, thus showing clearly that the circulation of the pepsin solution within the capillary tube was good. (See Table II.)

TABLE II.—*Lengths of Albumen Column Digested in Successive 24-Hour Periods.*

Case.	Length of column digested in ½ mm.						
	24 hours.	48 hours.	C.	72 hours.	C.	96 hours.	C.
I	8	16	8	24	8	32	8
II	9·5	19	9·5	28	9·5	..	..
III	5	10	5	15	5	20	5
IV	4	8	4	12	4	16	4
V	10	20	10	30	10	..	..

C represents the control tube.

The last finding also implies that a linear relationship exists between the length digested and the time allowed for the digestion to proceed. It occurred to us to apply this linear relationship to an examination of fluids of low peptic concentrations, so as to observe whether the square root law was applicable to such cases. For it has recently been argued that though the Schütz-Borissow law was invalid for high, it did apply to low concentrations (Nakajima, 1930). We therefore prepared pepsin solutions diluted to a point below the limit of accurate measurements in respect of length digested in the usual period of 24 hours. These were incubated for several days before readings were taken. The findings in this experimental series observed the same rule as the high peptic concentrations. (See Table III.)

TABLE III.—*Relation between Pepsin Concentration and Length of Albumen Digested, when Weaker Fluids were Incubated for Three Days.*

Dilution.	L.	L^2.	l^2.	L^3.	l^3.
1 : 16	6	36	36	216	216
1 : 32	5	25	18	125	108
1 : 64	4	16	9	64	54
1 : 128	3	9	4	27	27
1 : 256	2·5	6	2	15	13·5

L represents the length of albumen column digested in the Mett's tube. l^2 and l^3 are values representing the pepsin concentrations calculated from the 1 : 16 dilution on the basis of the square law and cube law respectively. The sample was taken from a case of carcinoma of the stomach, and was incubated for three days before the reading was taken.

SUMMARY.

The Mett method affords a simple and dependable means of estimating pepsin concentration if regard is paid to the following points :

1. The tubes to be prepared according to Christiansen's method and to be 1½ mm. bore.

2. Very weak pepsin solutions to be incubated several days before reading is taken.

3. The pepsin concentration to be taken as being proportional to the cube of the length digested, which provides a much more accurate evaluation than the Schütz-Borissow law.

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ON SERUM BILIRUBIN: I. THE DIAZO REACTION AS A QUANTITATIVE PROCEDURE.

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BILIRUBIN is a constant constituent of normal blood-serum and is in part responsible for its slightly yellow colour. Under certain pathological conditions the bilirubin content increases, with consequent increase in the yellow colour of the serum, and the measure of this increase is of value as an aid to diagnosis, and an indication of the severity of the diagnosed condition. Two methods of estimation are in use: a simple comparison of the colour intensity of the serum with an arbitrary yellow standard, the so-called icterus index (Meulengracht, 1920; Maue, 1922), and a direct estimation of the bilirubin content by means of Van den Bergh's diazo reaction (1913). For a method to be of most value as a routine procedure, it should conform to the three criteria—specificity, reasonable accuracy, and simplicity of technique. The icterus index owes most of its popularity to the very simple manipulative procedure involved, but on the other hand it is entirely empirical, the colour having no direct quantitative relationship to the content of pigment, and it is not specific, owing to the presence in serum of other yellow pigments, *e. g.* carotene, the content of which varies under certain conditions (White, 1931).

The diazo method of estimation is based upon the property discovered by Ehrlich (1883), in virtue of which bilirubin reacts with diazobenzene-p-sulphonic acid to form a highly coloured azo compound. The reaction is delicate, specific, and does not involve complicated manipulation, but it has not hitherto been universally regarded as of quantitative accuracy. Three main reasons account for this: In the first place, in carrying out the original Van den Bergh technique, some (and a variable amount) of the bilirubin is usually lost by adsorption on the serum proteins when these are precipitated by alcohol; the subsequent colour produced by addition of the diazo reagent to the supernatant liquid cannot therefore be related to the total amount of bilirubin originally present in the serum. Secondly, the observation of Van den Bergh and Müller (1916), that in some cases of jaundiced serum the bilirubin reacts immediately with the diazo reagent, while in others the reaction takes place very slowly or only after addition of alcohol—an observation which led them to suggest the existence in serum of two varieties of bilirubin; and the diversity of opinion which has arisen as to the origin, chemical nature, and clinical significance of these two forms, have led to a certain amount of scepticism as to the reliability of the procedure. Thirdly, Van den Bergh himself stated

that he has always called it an " estimation " and not a quantitative determination (1924), and until recently no further attempt has been made to correlate the intensity of colour produced, with the amount of bilirubin present.

Hunter (1930) reinvestigated the question and showed that in simple solution, and if due regard be paid to buffering, the coupling of bilirubin with the diazo reagent is complete, and produces a colour which is strictly proportional to the amount of bilirubin in solution. Hunter further claimed that this quantitative relationship also held (within limits) for bilirubin in serum, but the one example which he gave was not convincing. Since for practical purposes it is the behaviour of bilirubin in *serum* which is of paramount importance, the question seemed worthy of more extended study with a view to ascertaining the degree of accuracy which could be obtained under routine conditions.

In any method of estimation involving colorimetric comparison the choice of a suitable standard is of primary importance. When the substance to be determined can readily be obtained in a pure condition, the only point at issue is the most appropriate concentration to adopt for the standard solution ; on the other hand, when the substance, like bilirubin, is difficult to obtain, costly, and unstable in solution, some form of artificial standard is essential. Van den Bergh originally recommended an ethereal solution of iron thiocyanate, but this not proving altogether satisfactory, he replaced it with a solution of cobalt sulphate (McNee and Keefer, 1925), and this solution, prepared by dissolving 2·161 gm. of the anhydrous salt in 100 c.c. of water, is now in general use. For accurate work this is far from satisfactory, owing to the difference in tint between the red cobalt sulphate and reddish-violet azobilirubin colour, and the same objection must be made to the 1·9 per cent. cobalt sulphate solution suggested by Hunter. Nichols and Jackson (1930) prepared a series of solutions of varying proportions of cobalt chloride in hydrochloric acid solutions, standardized them against azobilirubin solutions of different strengths, and advocated the determination of unknown azobilirubin solutions by simple comparison with each of the series until a match is obtained. Obviously this method does not give the same degree of accuracy which can be obtained by the use of a colorimeter, but their claim that these solutions closely matched the azobilirubin colours suggested that the addition of hydrochloric acid to the cobalt sulphate solution might impart a violet tinge to the red colour. Such proved to be the case, and after a number of experiments with varying proportions of the salt and the acid, a solution was obtained which proved satisfactory, and has been used throughout this investigation. The details of its preparation are as follows :

1·3 gm. of anhydrous cobalt sulphate are dissolved in 50 c.c. of distilled water. To this is added gradually, with shaking and cooling, 40 c.c. of concentrated hydrochloric acid (sp. gr. 1·19), and the solution made up to 100 c.c. with distilled water. As the acid is added the colour of the solution changes to a bluish violet, which gradually reverts to a more reddish violet, the permanent hue. For this reason the solution should be prepared 24 hours before use, and kept well stoppered and out of contact with light. Prepared thus, the solution is apparently stable ; it has been tested repeatedly during a period

of three months, and has invariably shown the same azobilirubin colour value. Further, the colour intensity of the solution is proportional to the concentration of cobalt salt, and consequently weaker or stronger standards can be prepared by dissolving the appropriate amounts of the sulphate, adding 40 c.c. of concentrated hydrochloric acid, and making up to 100 c.c., although the colour is such that stronger standards are not recommended.

THE COLOUR VALUE OF THE COBALT SOLUTION IN TERMS OF BILIRUBIN.

In determining the azobilirubin colour value of this new cobalt solution, advantage was taken of the fact reported by Hunter that if the diazo reagent be buffered to a weekly acid reaction, the coupling with bilirubin is both immediate and complete, and consequently gives the maximum colour production. The solutions required were made up as follows, the materials used being all of analytical reagent quality.

Bilirubin Solutions.

An accurately weighed quantity of pure bilirubin (a pre-war Merck [Darmstadt] sample) was dissolved in pure chloroform in a 100 c.c. volumetric flask. When solution was complete the flask was made up to the 100 c.c. mark with more chloroform, and the contents mixed.

The Diazo Reagent.

Solution A.—1 gm. sulphanilic acid was dissolved in 1 litre of distilled water containing 15 c.c. concentrated hydrochloric acid.

Solution B.—0·375 per cent. sodium nitrite solution. Solutions A and B mixed in the respective proportions of 25 and 1 composed the diazo reagent.

Hunter's Buffer Solution.

6 gm. disodium hydrogen phosphate ($Na_2HPO_4.12HO_2$) were dissolved in water and the volume made up to 100 c.c. Equal quantities of this solution and the diazo reagent mixed together immediately prior to the test formed Hunter's buffered diazo reagent.

Using this buffered reagent, Hunter demonstrated that over a range of 0·1 to 0·01 mgm. bilirubin in 12·5 c.c. of solution, the amount of bilirubin in each solution and the colour produced from it were strictly proportional. His procedure consisted in taking quantities varying from 1 to 0·1 c.c. of a 0·01 per cent. chloroform solution of bilirubin, diluting in each case to 10 c.c. with 96 per cent. alcohol, and then adding 2·5 c.c. of the buffered reagent. Each solution in turn was transferred to one of the cups of a colorimeter, and read against a standard solution prepared in a similar manner from 0·5 c.c. of the bilirubin solution (*i. e.* containing 0·05 mgm. bilirubin). This procedure, slightly modified, was adopted in determining the colour value of the cobalt solution, which, placed in one of the colorimeter cups, was compared in turn against each member of a similar series of azobilirubin solutions. As a control, a 0·05 mgm. bilirubin solution was treated similarly. The only modifications introduced were in the proportions of the buffer mixture, 4 of the phosphate solution to 5 of the diazo reagent being found to be more suitable, and in carrying

out the coupling at a low (and constant) temperature. This investigation being carried out during the summer months when the temperature of the laboratory fluctuated considerably, it was felt that such variations would affect the time of coupling. A few preliminary experiments having demonstrated such to be the case, it was decided to carry out the standardization at the temperature of melting ice. This had the effect of slowing the rate of coupling, and a period of ten minutes had to be allowed for maximum colour development. On the other hand, the rate of fading was diminished to a much greater extent, so that the colour remained constant for periods of over one hour. Also under these conditions the buffered reagent remained colourless

TABLE I.—*Standardization of Cobalt Solution in Terms of Azobilirubin Colour Value.*

Bilirubin solution (mgm. per 100 c.c.).	Amount of solution taken (c.c.).	Amount of bilirubin taken (mgm.).	Colorimetric reading.		Bilirubin colour-value of cobalt solution (mgm.).
			Azobilirubin solution (mm.).	Cobalt solution (mm.).*	
10·8	0·2	0·0216	15	6·1	0·053
,,	0·3	0·0324	15	9·7	0·050
,,	0·4	0·0432	15	12·6	0·051
,,	0·5	0·0540	15	16·1	0·050
,,	0·6	0·0648	15	19·6	0·049
,,	0·8	0·0864	15	25·1	0·052
,,	1·0	0·1080	15	32·6	0·049
10·0	0·5	0·0500	15	15·0	0·050
18·0	0·5	0·0900	15	27·3	0·049
†30·0	0·2	0·0600	15	17·9	0·050
†54·0	0·1	0·0540	15	16·5	0·049
Mean value					0·050

TABLE II.—*Determination of Solutions of known Bilirubin Strength by Comparison of their Azo Colours, Using as Standards* (a) *the Cobalt Solution (equivalent to* 0·05 *mgm. bilirubin) and* (b) *the Azo Colour from* 0·05 *mgm. bilirubin.*

Amount of bilirubin taken (mgm.).	Colorimeter reading (*a*).		Amount of bilirubin calculated (mgm.).	Error (per cent.).	Colorimeter reading (*b*).		Amount of bilirubin calculated (mgm.).	Error (per cent.).
	Cobalt solution (mm.).	Azobilirubin solution* (mm.).			0·05 mgm. standard (mm.).	Azobilirubin solution (mm.).*		
0·0216	10	24·4	0·0205	−5·1	15	36·3	0·0207	−4·2
0·0324	10	15·2	0·0329	+1·5	15	22·4	0·0335	+3·4
0·0432	10	11·7	0·0427	−1·1	15	17·4	0·0433	*Nil*
0·0540	10	9·2	0·0543	+0·6	15	13·9	0·0539	,,
0·0648	10	7·6	0·0658	+1·6	15	11·2	0·0670	+3·4
0·0864	10	5·9	0·0847	−2·0	15	8·6	0·0872	+0·9
0·1080	10	4·6	0·1087	+0·6	15	7·0	0·1071	−0·8
0·0540	20	18·6	0·0537	−0·6	15	14·3	0·0525	−3·0
0·0600	20	17·0	0·0588	−2·0	..	..	..	..
0·0900	20	11·0	0·0909	+1·0	15	8·3	0·0904	+0·4
0·1620	..	..	..	..	30	9·4	0·1596	−1·5
Mean error (per cent.)				−0·5				−0·1
Average deviation from mean				±1·6				±1·8
Maximum deviation from mean				±4·6				±4·1

* Each figure represents the mean of several readings.

† Sample of pure bilirubin obtained from Messrs. Eimer & Amend, New York.

for at least thirty minutes, whereas, as Hunter also pointed out, at the temperature of the laboratory a distinct reddish colour was perceptible within ten minutes. Throughout this and all subsequent experiments, only standardized pipettes and volumetric flasks were used, and colorimetric comparisons were carried out in a dark room, the source of illumination being a colorimeter lamp.

The results obtained are shown in Tables I and II, and demonstrate that the cobalt solution is capable of being used with a good degree of accuracy as an artificial standard for the estimation of bilirubin, and that it has a colour equivalent to that produced by the action of the diazo reagent on 0·05 mgm. of bilirubin in 12·5 c.c. of solution, *i. e.* it has an azobilirubin colour value equivalent to 0·4 mgm. bilirubin per 100 c.c.

COMPARISON WITH VAN DEN BERGH'S TECHNIQUE.

In Van den Bergh's original paper he obtained the colour value of his artificial standard by taking 1 c.c. of a 0·005 per cent. chloroform solution of bilirubin, evaporating on the water-bath, taking up the residue in 10 c.c. of a solution containing 3 gm. sodium chloride and 0·6 gm. sodium bicarbonate per litre of 70 per cent. alcohol, and acting upon this with 2·5 c.c. of the diazo reagent. Hunter quotes Fischer and Barrenscheen (1921) as stating that the residue after evaporation is not completely soluble in van den Bergh's alcoholic solution—a finding which Hunter confirmed. Notwithstanding, van den Bergh must have succeeded in this, and it was therefore of interest to attempt to reproduce this result. This was accomplished by evaporating the chloroform solution in a test-tube. On removing the test-tube from the water-bath and cooling, sufficient vapour condensed to furnish a drop of liquid which partially redissolved the residue. Addition of 0·5 c.c. of the chloride-bicarbonate-alcohol mixture and slight warming brought the residue into solution, and it was then diluted with a further 9·5 c.c. of the mixture, and treated with the reagent. After ten minutes the resultant colour was compared in the colorimeter against the same amount of bilirubin treated according to Hunter's technique, and also against the new cobalt standard. Although the colour was much more blue than that of either of the standards and therefore difficult to compare, the intensity was about the same in all three cases. This indicated that if allowance be made for a greater limit of error due to the comparison of different shades of colour, the same amount of azobilirubin was produced in Van den Bergh's alcoholic solution as in Hunter's more strongly buffered solution, confirming therefore the quantitative basis of Van den Bergh's standardization of his artificial solution in terms of bilirubin.

THE ESTIMATION OF BILIRUBIN DISSOLVED IN SERUM.

It is not the intention to discuss here the many theories which have been evolved purporting to explain the existence in serum of two forms of bilirubin. No theory, however, can be accepted which does not take into account the fact that icteric serum as generally obtained is a clear yellow liquid, indicating that, whatever may be the form or combination in which the bilirubin exists in the liquid, it is apparently in true solution. Hunter added 0·1 c.c. of a chloroform solution of bilirubin to serum, followed by the diazo reagent and 96 per

cent. alcohol, and reported that the amount of colour developed was the same as that produced by the addition of the buffered diazo reagent to a similar amount of bilirubin solution, diluted with alcohol to the same final volume. Since the bilirubin was never actually in solution in the serum, all that can be deduced from this experiment is that the addition of serum does not interfere with the estimation of bilirubin in a chloroform-alcohol-water mixture. In the experiments now reported, the procedure consisted in pipetting quantities varying from 0·1 to 1 c.c. of a chloroform solution containing a known quantity of bilirubin into clean dry test-tubes, and evaporating the solvent on the water-bath, the last traces being removed by suction. The residue was dissolved in 1–2 drops of 2 per cent. sodium carbonate solution, 5 c.c. of serum added immediately, and the whole well mixed. In every case a clear solution was obtained. Fresh ox serum was used after it had been treated with decolorizing charcoal (norite), centrifuged, and filtered first through kieselguhr, and then (by gravity) through hardened filter-paper; on testing, it was found to be free from bilirubin. In the majority of cases the estimations were carried out both by Van den Bergh's original procedure, and by the modification suggested by Thannhauser and Andersen (1921), the colour produced by the interaction with the diazo reagent being compared in the colorimeter against the new cobalt standard, and the results expressed in mgm. bilirubin per 100 c.c. of serum.

In Van den Bergh's procedure the proteins are precipitated by the addition of 2 c.c. of 95 per cent. alcohol to 1 c.c. of serum. After centrifuging, 1 c.c. of the clear supernatant fluid is diluted with 0·5 c.c. of 95 per cent. alcohol, 0·25 c.c. of the freshly prepared diazo reagent added, and the resultant colour compared against the standard.

Calculation.—The addition of 2 c.c. of alcohol to 1 c.c. of serum gives a dilution of 20/7 (owing to the contraction due to alcohol). This liquid is further diluted by 7/4, and therefore the total dilution is 5. The cobalt solution has a colour value equivalent to 0·4 mgm. bilirubin per 100 c.c. The bilirubin content of the serum is therefore calculated from the formula:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 5 \times 0{\cdot}4 = \text{mgm. bilirubin per 100 c.c. serum.}$$

Thannhauser and Andersen's modification was introduced to avoid loss of bilirubin by adsorption on the protein precipitate. In this procedure, 0·5 c.c. of the diazo reagent is added directly to 1 c.c. of serum, followed, after an interval of 15 minutes, by 2·5 c.c. of 95 per cent. alcohol and 1 c.c. of a saturated solution of ammonium sulphate, mixing after each addition. On centrifuging, the mixture separates into three layers, the lowest being colourless ammonium sulphate solution, then a layer of precipitated protein, and superimposed upon that, a clear coloured layer. The latter is removed and compared against the standard in the colorimeter.

Calculation.—Although the serum is apparently diluted five times, Thannhauser and Andersen took 1 in 4 to be a more accurate figure for the dilution, since only 0·2 to 0·3 c.c. of the ammonium sulphate solution is taken up by the other constituents, and they considered that this was compensated for, approximately, by the volume of the precipitated proteins. This dilution factor has been called in question by Hall (1927) and others, who maintain

that all the azobilirubin is contained in the coloured layer above the precipitated proteins, and that the volume of this layer, divided by the amount of serum taken, constitutes the dilution factor. When the amount of serum is 1 c.c., the volume of the coloured layer is generally about 3 c.c., giving bilirubin values about 25 per cent. less than those obtained by the original calculation. This modified form of calculation has been adopted by Hawk and Bergeim in the latest edition of their text-book (1931), although, as far as the writer is aware, no proof has been advanced that the older form is incorrect. Admittedly the way in which Thannhauser and Andersen arrive at the dilution figure is of rather an arbitrary nature, and yet, as will be shown, their dilution figure is approximately correct.

It is commonly stated that the protein layer is colourless, but in the experiments now reported, and also in a large number of estimations on jaundiced sera, it was noticed that this was not the case, the protein layer being coloured to a greater or less extent, depending upon the depth of colour in the supernatant layer. The lower (ammonium sulphate) layer was invariably quite colourless ; it therefore contained no azobilirubin and could be disregarded. This left a volume of coloured liquid containing a precipitate, separated by centrifugal force into liquid and solid phases. The assumption that all the azobilirubin is in the supernatant liquid implies that the protein precipitate is so compressed as to afford *complete* separation into two phases, similar to the separation of two immiscible liquids — a consummation very doubtful of attainment with the usual laboratory type of centrifuge. It follows, therefore, that the solid phase must contain an admixture of the coloured liquid phase, which is in accordance with the writer's observation. This solid phase consists mainly of proteins which had, when in solution, formed part of the serum volume, and without entering into the question of the relative volumes of a molecule in the solid form and in solution, it seems reasonable to suppose that if this precipitated protein volume be ignored, then the supernatant liquid represents the dilution of the *deproteinized* serum, whose volume must be less than 1 c.c. Taking this into account, it appeared that a smaller error would be made if the dilution were represented by the ratio of the total volume occupied by the precipitate and supernatant liquid to the volume of serum taken. This method of arriving at the dilution factor is similar to that used by Van den Bergh in the first step of his quantitative estimation, and also to that which forms the basis of the well-known Folin-Wu method of blood analysis.

In carrying out estimations by the Thannhauser and Andersen method, therefore, a graduated centrifuge tube was used, and after centrifuging for a few minutes, the volume of the ammonium sulphate layer was read and subtracted from the total volume, and a portion of the coloured supernatant liquid compared in the colorimeter against the cobalt standard. The bilirubin content was calculated from the formula :

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{dilution factor} \times 0{\cdot}4 = \text{mgm. bilirubin per 100 c.c. serum,}$$

where the dilution factor $= \dfrac{\text{Total vol.} - \text{vol. ammonium sulphate layer}}{\text{Vol. of serum taken.}}$

It should be noted that when the volume of serum taken was 1 c.c., the dilution factor was usually 4.

The results are shown in Table III.

TABLE III.—*Bilirubin Determinations by* (a) *the Van den Bergh Procedure, and* (b) *Thannhauser and Andersen's Modification, on Sera of Known Bilirubin Content.*

Known bilirubin content of serum (mgm. per 100 c.c.).	Bilirubin determined (*a*) (mgm. per 100 c.c.).	Error (per cent.).	Bilirubin determined (*b*) (mgm. per 100 c.c.).	Error (per cent.).
1·00	0·90	−10·0	0·93	−7·0
1·08	0·87	−19·4	1·16	+7·4
1·40	..	..	1·34	−4·3
1·80	1·43	−20·5	1·90	+5·5
1·80	..	..	1·89	+5·0
1·80	..	..	1·90	+5·5
2·00	1·69	−15·5	2·00	*Nil*
2·00	..	..	2·05	+2·5
2·16	1·73	−20·0	2·10	−2·8
2·60	2·10	−19·2	2·50	−3·8
2·60	..	..	2·60	*Nil*
3·60	3·17	−12·0	3·72	+3·3
3·60	2·89	−19·7	3·73	+3·6
3·60	2·77	−23 0	3·43	−4·7
4·30	..	..	4·10	−4·7
4·80	3·90	−18·7	4·92	+2·5
10·80	9·65	−10·6	10·66	−1·3
11·20	10·05	−10·3	11·31	+1·0
Mean error (per cent.)		−16·6	.. ..	+0·4
Average deviation from mean		± 4·1	.. ..	±3 6
Maximum deviation from mean ..		± 6·6	.. ..	±7·4

The results show that by the employment of Thannhauser and Andersen's technique and the form of calculation described, the bilirubin content of serum can be determined with reasonable accuracy. With the original Van den Bergh method, on the other hand, consistently low results are obtained, and it cannot be regarded therefore as a satisfactory method of quantitative determination. This is in agreement with the views expressed by several investigators. Shay and Schloss (1929) reported the results of 28 cases of different types of jaundice, in which the bilirubin content of the serum, as determined by Van den Bergh's procedure, was from 17 to 86 per cent. less than the figure obtained by the Thannhauser and Andersen technique. This shows a much greater variation than the corresponding figures in Table III, but it must be remembered that we are still in ignorance of the form or forms in which bilirubin is present in cases of jaundiced sera, and how this may influence the adsorption of the pigment upon the precipitated proteins. In the present series of experiments bilirubin was present in one form only—as a sodium salt—and in view of the fairly consistent nature of the results obtained, and the fact that in icteric sera of clinical origin, some of the bilirubin is most probably present in combination with base, it seems likely that in at least some types of jaundice the bilirubin is present in the serum in more than the one form of chemical combination. It is hoped to present further and more conclusive evidence of this in a subsequent communication.

These considerations do not, however, invalidate the use of Thannhauser and Andersen's modification as a means for the quantitative estimation of bilirubin in serum, from whatever source, since this technique avoids the loss

by adsorption of unacted-upon bilirubin. The form of calculation used in these experiments has the advantage that smaller amounts of serum may be taken without varying the amounts of the other constituents, or conversely, the amount of serum may be kept constant and the other constituents varied without affecting the result, provided there is sufficient diazo reagent to react with all the bilirubin, and sufficient alcohol and ammonium sulphate to precipitate the proteins completely. The amount of serum can therefore be so adjusted that the azobilirubin colour produced is within the range of comparison of the artificial cobalt standard, although the smaller the amount of serum taken the less is its power of buffering the acidity of the diazo reagent, with a consequent tendency towards a blue tinge in the azobilirubin colour. Although this detracts somewhat from the facility with which colorimetric comparison can be made, that it does not affect the accuracy to any appreciable extent the following experiments show (Table IV):

TABLE IV.—*Duplicate Bilirubin Determinations on Sera from Cases of Clinical Jaundice.*

Case No.	Amount of serum taken (c.c.).	Amount of alcohol added (c.c.).	Bilirubin content (mgm. per 100 c.c.).	Mean value.	Deviation from mean (per cent.).
39	0·5	2·5	11·2	11·5	2·6
	0·25	2·5	11·8		
44	0·5	2·5	9·0	8·9	1·1
	0·25	2·5	8·8		
52	0·2	2·5	23·4	23·05	1·5
	0·1	2·5	22·7		
53	0·5	2·5	17·0	17·0	*Nil*
	0·2	2·5	17·0		
56	1·0	2·5	8·0	8·2	2·4
	0·5	2·5	8·4		
57	1·0	2·5	5·8	5·7	1·8
	1·0	3·0	5·6		
59	1·0	2·5	1·7	1·7	*Nil*
	1·0	3·0	1·7		

SUMMARY.

It has been shown that when the diazo reagent is permitted to react with bilirubin dissolved in blood-serum, the colour produced is strictly proportional to the amount of bilirubin in solution. Using the Thannhauser and Andersen technique, and a revised form of calculation, it is therefore possible to determine with reasonable quantitative accuracy the bilirubin content of icteric sera.

Although evidence is given supporting the quantitative basis of Van den Bergh's experiments, his procedure for the estimation of bilirubin in serum yields consistently low results, due, presumably, to the adsorption of bilirubin on the proteins precipitated by alcohol.

Details are given of a new cobalt solution capable of being used with accuracy in place of pure bilirubin, as a standard solution.

This is the first part of an investigation made possible by the assistance of a grant from the Trustees of the Banting Research Foundation, to whom I desire to express my gratitude.

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INFLUENCE OF THE BACTERIAL CELL ON THE PHAGE-ANTIPHAGE REACTION.

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THERE is evidence in the literature (Wright and Douglas, 1904 ; Rous and Jones, 1916) that bacteria and red blood-cells, when they have been taken up by living animal cells, are thereby protected from the action of external injurious agents, including immune sera. Similarly it appears that certain viruses such as herpes (Andrewes, 1930), when once they have penetrated within a living susceptible cell, can no longer be neutralized by antiserum. Experiments are here recorded showing how far similar conclusions are applicable to a strain of bacteriophage in its relations with a neutralizing antiserum and with *Bacillus coli*.

Antiphage Serum.

All workers agree that it is possible by immunizing rabbits to obtain a phage-neutralizing antibody which is distinct from any antibacterial antibody, and is more or less specific for the particular phage used for immunizing. The antibody is apparently effective in the absence of complement and unites directly with the phage. Whether or not the phage-antibody union is a stable one is disputed ; it is hoped to publish shortly a paper dealing with the nature of the reaction.

Experimental Methods.

The strains of phage and of *B. coli* were those employed in an earlier study (Andrewes and Elford, 1932). The phage was commonly active in a dilution

of 10^{11}. Antisera were obtained by injecting rabbits with phage filtrates, at first intravenously, later subcutaneously. Four injections were given at 4-day intervals, in doses rising from 1 to 5 c.c. ; the rabbits were rested for a month, and then another similar course was given. Later, whenever serum was required, rabbits received subcutaneous injections of 5 c.c. of phage filtrates on two successive days, and blood was taken 5 days later. One rabbit thus treated has continued to yield an active serum over a period of two years. Sera from the later bleedings were relatively poor in agglutinins for *B. coli*. Control experiments with sera absorbed with *B. coli* indicated that antibacterial antibodies played no part in the phenomena described in this paper.

Experimental Results.

Phage diluted 1 : 1000 when left in contact with an equal volume of undiluted immune serum was completely inactivated within 24 hours even at 0° C. ; at higher temperatures neutralization was of course more rapid. It was desired to find out whether phage, if it were previously given the opportunity to attach itself to or penetrate living *B. coli*, would be protected from this neutralizing action. Long contact of phage and organisms before the addition of antiserum was not considered necessary, as it is generally agreed that phage is taken up by a susceptible organism very rapidly. It will appear that temperature had an important influence on the outcome of the experiments.

Experiment (1) : *Attempted Neutralization at 0° C.*

A mixture was made of 1 c.c. of a 1 : 1000 dilution of phage and 1 c.c. of a culture containing about 10^8 *B. coli*. After contact for 5 minutes at room temperature to allow the organisms to take up the phage the mixture was cooled to 0° C., and 2 c.c. of antiserum cooled to 0° C. were added ; the tube was then placed in the ice-box. Even after standing in the cold for 5 days, the phage in this mixture was found to be undiminished in titre so far as the accuracy of the method of titration in broth could detect. The same dilution of phage left in contact with immune serum at 0° C. in the absence of *B. coli* was, as already mentioned, completely inactivated in 24 hours. It thus appears that living *B. coli* can protect the phage from the action of antiserum at 0° C.

Experiment (2) : *Neutralization at 37° C.*

The experiment was repeated ; but this time, as soon as the antiserum had been added to the phage-coli mixture, the tube was placed in the 37° C. incubator. At intervals 0·1 c.c. samples were removed from the tube and spread on agar plates along with three drops of a culture of *B. coli* ; no lysis occurred in the tube, and the plates sown with successive samples showed, when they were examined next morning, that the quantity of phage in the tube had steadily diminished, until finally it disappeared altogether within 24 hours (see Fig. 1). Such a result was only obtainable when undiluted serum was used.

Experiment (3): *Neutralization at 20° C.*

At room temperature (20° C.), also, the undiluted serum was able to effect the neutralization of phage in a phage-coli mixture; it was, however, less effective than at 37° C. In one experiment three lots of phage diluted 1 : 10,000 were mixed with a culture of *B. coli* and held 20 minutes at 20° C.; then an equal volume of antiserum diluted 1 : 2 was added to each mixture. These were held at different temperatures for 24 or 48 hours, and then tested on plates for the presence of phage as shown in Table I. Probably because 1 in 2 antiserum was used, neutralization was not quite complete in the tube held at 37° C., even after 24 hours.

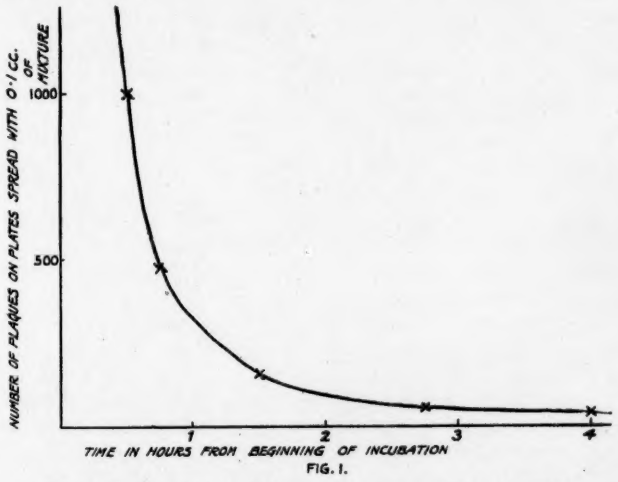

FIG. 1.

Neutralization of phage in a phage-organism mixture at 37° C.

TABLE I.—*Experiment* (3): *Neutralization of Phage in Phage-Organism Mixtures at Different Temperatures.*

Contact of antiserum (1 : 2) with mixture of *B. coli* and dilute phage.	Number of plaques on plate sown with 0·1 c.c.
24 hours at 0° C.	Confluent lysis.
24 ,, 20° C.	140
48 ,, 20° C.	63
24 ,, 37° C.	14

Experiments 2 and 3 give results opposite to those which might have been expected by analogy with the findings with herpes virus. Although the phage had a plentiful opportunity to infect the bacterial cell before the immune serum was added, yet the antiserum was not prevented from neutralizing it. Possible reasons for this discrepancy will be discussed later.

Experiment (4): *Experiment with Heat-killed Organisms.*

Living *B. coli* seemed able to protect phage from neutralization only at 0° C. It remained to show whether heat-killed *B. coli* could similarly protect

it at that temperature. *B. coli* were killed by heating them to 60° C. for 1½ hours. It was found that phage diluted 1 : 10,000 and mixed with these killed organisms was completely inactivated by antiserum after 24 hours at 0° C., just as it was in the absence of bacteria. One might interpret this result as confirming the experiments of Rous and Jones (1916), who found that dead cells had lost the power of protecting bodies within them from external injurious agents. But another explanation seems equally feasible. Phage may be adsorbed on to dead bacteria, and according to Krueger (1931) this adsorption is irreversible ; the phage cannot be freed to attack living organisms. It is possible, therefore, that in Experiment (4) some of the phage was adsorbed on to the killed organisms and was inactivated for that reason, while that which was not adsorbed was neutralized by the immune serum.

Experiment (5) : *Effect of Sodium Citrate.*

Immune serum was added to a *B. coli*-phage mixture, together with sodium citrate in quantity sufficient to bring its final concentration up to 0·5 per cent. The phage was gradually neutralized, just as in the absence of citrate. The concentration of citrate used, as described in another paper (Andrewes and Elford, 1932), is sufficient to inhibit the multiplication of this strain of phage and the lysis by it of *B. coli*. Neutralization can thus occur though no lysis of organisms is taking place.

DISCUSSION.

How does it happen that when bacteriophage has been taken up by a susceptible cell it is not protected at 37° C. from the action of immune serum, as many viruses seem to be in similar circumstances ? Four possible explanations may be mentioned :

(1) Many will be reluctant to believe that the antibody can penetrate the bacterial cell-wall and attack the phage within, for a great deal of evidence exists to show that antibodies act at the surface of bacteria.

(2) It is generally assumed that bacteriophage multiplies within bacteria and does not act only at their surface ; but this assumption may be altogether wrong. The protection against antiserum which the bacteria have been shown to afford at 0° C. does, it is true, suggest that the phage is not only on the bacterial surface, but actually within. This argument is not, however, irrefutable ; the adsorption of the phage to the bacteria may be so much firmer at low temperatures that the antiserum cannot snatch it away.

(3) In the course of multiplying in a culture, phage must be constantly coming out of the bacterium " into the open." This is likely to be true whether or not d'Hérelle's picture of the course of events is correct. D'Hérelle believes that a limited number of bacteria may be infected at first and that phage may multiply in them and lyse them, being liberated in the process. If this were true much of the phage could be neutralized by antiserum as it was thus set free and before it could infect many further organisms.

There is, however, an array of evidence brought forward by Krueger and Northrop (1930) and others indicating that this series of events does not take place, and that no lysis occurs at all until the phage has reached a fairly high

titre. My colleague, W. J. Elford, and I certainly failed to obtain any evidence, either by studying the turbidity of cultures, or by watching organisms by dark-ground illumination, that any bacteria at all were lysed, until the critical moment at which lysis became almost universal through the culture (Andrewes and Elford, 1932).

A further fact makes it difficult to explain the results in this paper in terms of d'Hérelle's view. As already mentioned, sodium citrate inhibits the lytic action of the phage under study. If, then, phage is neutralized only when it is liberated by lysis of an infected organism, citrate, which inhibits that lysis, should also inhibit neutralization. Experiment 5 revealed that it did not do so.

(4) Yet another possible explanation is afforded by the work of Krueger and Northrop (1930) and Krueger (1931). They believe that when phage is in contact with living organisms there is an equilibrium—they refer to it as a partition coefficient—between the intra- and extra-cellular phage. I have not undertaken the somewhat extensive investigations necessary to confirm or refute this suggestion, but it is at any rate a view which is consistent with the facts of the experiments recorded here. For if immune serum were to neutralize all the extra-cellular phage, more would have to leave the bacteria in order to restore the equilibrium ; more would then be neutralized, and so on. When the rate of neutralization was more rapid than the rate of multiplication, the phage would gradually diminish in quantity ; such conditions might be presumed to hold in Experiment (2)—that conducted at 37° C. The lower temperature in Experiment (1) might reasonably be expected to delay the restoration of the equilibrium between intra- and extra-cellular phage, though the contrasts found between the experiments at 0° and 37° C. seem almost too great to be explained so simply. Possibly the surface of the organism is relatively impermeable to phage at 0° C.

The experiments reported seem of interest in connection with the statement (Muckenfuss, 1928) that cultivation in antiphage serum will " cure " a lysogenic bacterium.

CONCLUSIONS.

1. Coli-phage which has been taken up by living *B. coli* is protected from the neutralizing action of antiphage serum at 0° C.

2. At 37° C. and at 20° C. this protective action is no longer seen ; possible explanations of this are discussed.

3. Heat-killed *B. coli* exercise no protective action at 0° C.

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OBSERVATIONS ON THE GROWTH REQUIREMENTS OF VACCINIA VIRUS *IN VITRO*.

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DURING the course of work on the cultivation of vaccinia virus a number of facts have accumulated which are germane to the problem of determining the requirements for growth of the virus *in vitro*. It is now generally accepted that multiplication of cells in tissue culture is not a necessary condition for successful cultivation of the virus. It is also well established that cultures of the virus are readily obtained in the presence of certain minced tissues in Tyrode's solution, with or without serum. Under these circumstances the tissue cells survive for a time. Whether the virus will grow in the absence of tissue cells is an open question. Eagles and McClean (1930, 1931) have reported series of cultures in a cell-free extract of adult rabbit kidney and Tyrode's solution. Rivers (1931) is of opinion that surviving susceptible cells are necessary. The experiments to be discussed here are interpreted in relation to this question.

EXPERIMENTAL.

Two strains of virus have been used. Strain I has been cultivated since October, 1928. For some of the later experiments a recently cultivated strain of neurovaccine was used—Strain II. The routine method of cultivation, titration and storage was that described in an earlier paper (Maitland and Laing, 1930). The medium was minced adult rabbit kidney and Tyrode's solution, with 30 per cent. of fresh rabbit serum. In some of the recent cultures serum has been omitted. Cultures were incubated in Carrel flasks at 37° C., 2 c.c. of medium in each flask. For titration the contents of a flask were ground with sand, centrifugalized, and 0·2 c.c. of various dilutions of the supernatant fluid inoculated intradermally into the shaved back of a rabbit. This method of cultivation has served throughout as a control.

In all the experiments, whenever a modified culture was titrated, an exactly comparable control culture was also titrated on the same rabbit. The growth of the virus in the controls has continued to be so consistent that differences observed between control and experimental flasks have been clear cut and definite.

The Omission of Serum from the Medium.

The observation of Li and Rivers (1931) and Rivers (1931) that serum could be omitted from the minced tissue medium has been confirmed. They cultivated the virus in a mixture of minced chick embryo and Tyrode's solution. We have found minced adult rabbit kidney and Tyrode's solution to be as satisfactory as our original medium, which had the addition of 30 per cent.

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rabbit serum. This modification did not appear to alter adversely the viability of the culture-virus during storage. It has been subcultured after storage at 5° C. for 22, 37 and 55 days.

The Saline Constituents of the Medium.

The Tyrode's solution has been made according to the formula given by Fischer (1925): NaCl 8·0 gm., KCl 0·2 gm., $CaCl_2$ 0·2 gm., $MgCl_2$ 0·2 gm., $NaHCO_3$ 1·0 gm., glucose 1·0 gm., NaH_2PO_4 0·05 gm., water 1000 c.c. It was sterilized by filtration through a Seitz filter.

The substitution of a buffered phosphate solution for Tyrode's solution has prevented multiplication of the virus (Strain I). In these experiments M/50 phosphate solution pH 7·6 (K_2HPO_4 and KH_2PO_4) was used in place of Tyrode's solution. Although regular increase of virus occurred in the control flasks, in no instance has the virus multiplied in the phosphate medium; as incubation proceeded the amount of living virus in the flasks decreased. Similar results were obtained with M/15 phosphate pH 7·6. This difference was not attributable to differences in pH, as the reaction of the Tyrode's solution was also 7·6. Nor was the phosphate itself injurious to survival of the virus at 37° C.; virus survived at least as long in the phosphate as in the Tyrode's solution. The difference in these two media in promoting and failing to promote the multiplication of virus depended apparently on a difference in saline constituents. In seeking an explanation two possibilities suggest themselves: either that the phosphate solution was deficient in a proper balance of certain ions required by the virus for growth, even though serum was present, or that the kidney cells were able to survive in a mixture of serum and Tyrode's solution in which the ionic composition was favourable, but were unable to do so in the unbalanced phosphate-serum mixture. It is not possible to be sure which of these possibilities is correct, but it is at least probable that the virus did not grow in the phosphate mixture because the tissue cells did not survive in it.

Cultivation in Test-tubes.

The depth of the medium in a Carrel flask is from 1 to 2 mm. Experiments were made in which half of the inoculated medium (minced kidney, Tyrode's solution and 30 per cent. serum) was distributed into flasks and half into tubes, 2 c.c. to each. In the tubes the depth of medium was approximately 15 mm. After incubation at 37° C. for periods varying from 2 to 7 days, the contents of a flask and a tube were titrated on one rabbit. The virus (Strain I) multiplied consistently in the flasks, but in the tubes it decreased progressively as incubation continued. The only difference between the conditions in the two sets of cultures was the depth of fluid covering the tissue and the aggregation of the tissue particles into a mass at the bottom of the tube in contrast to their being spread out just beneath the surface of the medium in the flask. The tissue in the tube would receive a poorer supply of oxygen than that in the flasks, and would, therefore, be expected to survive for a shorter time. This view was supported by measurements of respiratory activity of the tissues, as noted in another section. There was thus in these experiments evidence that failure of the tissue to survive was associated with absence of multiplication of the virus.

Incubation of the Medium before Inoculation.

Rivers, Haagen and Muckenfuss (1929) have shown that some of the pieces of tissue taken from Carrel flasks in which vaccinia virus was growing in a medium of minced hen's kidney, Tyrode's solution and serum, were still alive after 5 days' incubation; when transferred to a fresh plasma-clot pabulum on cover-slips proliferation of cells occurred.

We have made some observations on the period for which, prior to inoculation, minced rabbit kidney could be incubated at 37° C. in Tyrode's solution, or in Tyrode's solution and 30 per cent. rabbit serum, and still serve for cultivation of virus (Strain I). The medium was prepared and distributed into Carrel flasks, as was done in setting up cultures. Some flasks were inoculated at once, others after incubation for 2, 3, 5, 7 and 10 days. Titration of the contents of a flask immediately after it was inoculated gave a baseline by which to judge whether the virus had increased in others during further incubation.

A medium containing kidney, Tyrode's solution and serum and incubated for 2 days was suitable, and good growth of virus was obtained in all flasks. After incubation for 5 days some flasks were still suitable, but in others no growth of virus occurred. When serum was omitted, and the medium consisted of minced kidney and Tyrode's solution, it could be incubated for 2 or 3 days only without impairment; incubation for 5 days or longer rendered it useless for cultivation of the virus.

The length of time for which preliminary incubation of the medium could be made, without abolishing its property of providing suitable conditions for growth of vaccinia virus, corresponded with the time during which tissue cells would be expected to survive in it. Tests of the respiratory activity of the tissue surviving under identical conditions (in Tyrode's solution and serum) indicated that respiration could be detected for 3 days.

The Respiratory Activity of Surviving Cells.

These experiments constitute Mr. Lyth's contribution to the work, and form a preliminary survey of the relation between the respiratory activity of surviving cells and the conditions required for growth *in vitro* of vaccinia virus. Maitland and Laing (1930) had observed that whereas minced rabbit kidney or testis in Tyrode's solution and 30 per cent. serum served for cultivation, minced liver or spleen did not. The respiratory activity of these tissues was investigated after incubation in the Tyrode-serum solution in Carrel flasks at 37° C. for various periods. The conditions of incubation were identical with those employed for culture except that virus was not present. The technique of Warburg was followed, allowance being made for the serum in the mixture, and the fact that the tissues were finely minced with scissors instead of being cut into thin slices.

In general, these preliminary results may be summarized as follows: The tissues may be placed in the following order as regards their respiratory activity from day to day, beginning with the most active tissue: Testis, kidney, liver, spleen. Kidney had a definitely higher value than liver, whilst spleen respired very little, if at all. Kidney was not observed to respire after the third day.

Kidney in a test-tube, when the depth of fluid was 15 mm., did not respire after it had been incubated for 24 hours.

As far as these experiments go, they indicated (1) a correlation between the surviving respiratory activity of different tissues and their suitability to promote the growth of vaccinia virus ; (2) a correlation between the period for which a tissue (kidney) retained at 37° C. its property to promote the growth of virus and the period for which it respired ; and (3) a correlation between the unsuitability of kidney in test-tubes under a layer of fluid 15 mm. deep and the absence of respiration.

Attempts to Cultivate the Virus without Tissue-cells.

It may be noted here that the cell-free extracts in which Eagles and McClean (1931) obtained cultures, were prepared by adding 3 to 4 c.c. of Tyrode's solution to a finely minced rabbit kidney and centrifugalizing the mixture for 20 minutes. The supernatant fluid was the extract. In some experiments this was filtered to ensure the complete removal of cells. Filtered or unfiltered extract, with or without serum, served as the medium.

In Table I we have summarized the results of attempts to cultivate the virus in a cell-free medium. They were uniformly unsuccessful. In each experiment a comparison was made between a kidney extract medium and a control medium of minced kidney in the same solution. In no instance has anything more than survival of the virus been detected in the kidney extract, and the amount of virus that survived, as well as the period of its survival, depended directly upon the amount of virus in the flasks before incubation (at 37° C.). A few of the experiments require special comment. The titre of the virus before inoculation was calculated from an earlier titration of the inoculum and the dilution made in setting up the cultures, which does not allow for any deterioration of the virus during its storage. In the first three experiments there was but little indication of growth of virus in the medium containing cells, but the amount of virus inoculated was almost equal to the limiting concentration of virus in some of our cultures after incubation, so that this result was not unexpected. It is more noteworthy that in the extract medium, even after a heavy inoculation, the virus not only failed to grow, but that during incubation the amount of virus progressively decreased and was always much less than in the medium containing cells. For experiment No. 66, the inoculum was an emulsion of rabbit testis which had been infected with a strain of neurovaccine. It is of interest that the virus did not multiply in the kidney-cell medium in this (the first) culture, also that it survived more readily than in the kidney extract. In the second culture, in kidney cells, the inoculum being taken from the 4-day flask of experiment No. 66, the virus increased. Experiment No. 70 represents the third culture of the strain in series.

Unsuccessful attempts have been made to subculture virus that had survived in kidney extract and serum by transferring it to fresh similar medium, and in some instances to medium containing cells. In Experiment 77 (Table I) the contents of the flask that had been incubated for 2 days were diluted 1 in 4 in making the subcultures. No virus was detectable in the subcultures in extract and serum on the third, fifth and seventh days of incubation, whilst

TABLE I.—*Comparison of Survival of Virus in Kidney Extracts with Growth of Virus in a Medium containing Surviving Cells.*

No. of experiment.	Strain of virus.	Titre before incubation.	Nature of medium.	Titre after incubation. 2 days.	3 days.	4 days.	5 days.	7 days.
55	I	1/19000	Kid. ext. ser. Tyr.	..	..	..	1/125	..
			,, cells ser. Tyr.	..	..	..	1/15625	..
56	I	1/19000	,, ext. ser. Tyr.	..	1/625	..	1/125	1/5
			,, cells ser. Tyr.	..	1/3125	..	1/78125	1/15625
57	I	1/19000	,, ext. ser. Tyr.	1/625	..	1/5	..	1/5
			,, cells ser. Tyr.	1/15625	..	1/78125	..	1/15625
66	II	1/15625	,, ext. Tyr.	1/25	..	*Nil*	..	*Nil*
			,, cells Tyr.	1/15625	..	1/3125	..	1/25
70	II	1/6	,, ext. Tyr.	*Nil*	..	*Nil*	..	*Nil*
			,, cells Tyr.	1/625	..	1/3125	..	1/15625
71	II	1/150	,, ext. ser. Tyr.	..	1/5	..	*Nil*	*Nil*
			,, cells ser. Tyr.	..	1/625	..	1/78125	1/390625
74	I	1/30	,, ext. Tyr.	*Nil*	..	..	*Nil*	*Nil*
			,, cells Tyr.	1/25	..	..	1/3125 (C)	1/15625 (C)
76	II	1/150	,, ext. Tyr.	*Nil*	..	*Nil*	..	*Nil*
			,, cells Tyr.	1/125	..	>1/78125	..	>1/390625
77	II	1/780	,, ext. ser. Tyr.	1/25	..	..	*Nil*	..
			,, cells Tyr.	1/3125	..	..	1/15625	..

Kid. ext. ser. Tyr. = Kidney extract, serum and Tyrode's solution. > = not an end-point. (C) = bacterial contamination. Each figure represents the titration of the contents of one flask (0·2 c.c. intradermally in rabbits).

in the medium containing cells there was a five-hundredfold increase. In two other experiments not shown in Table I, virus (Strain II) that had survived for 4 days at 37° C. in kidney extract and serum was subcultured to the same medium, and was not detectable in subcultures incubated for 5 days at 37° C. There has been no evidence, therefore, that the virus adapted itself to a cell-free medium.

The extracts for these experiments have been made in various ways. For the first 3 experiments the minced kidney medium was prepared as for culture, incubated in Carrel flasks for 3 hours and centrifugalized. The supernatant fluid formed the extract medium. For the other experiments kidney was minced and ground with sand or glass in a mortar, Tyrode's solution added and the mixture centrifugalized, or the minced kidney was mixed with Tyrode's solution and centrifugalized. In no instance was the supernatant fluid filtered. When serum was added the amount was always 30 per cent. of fresh rabbit serum from defibrinated blood.

DISCUSSION.

All the evidence we have obtained points to the conclusion that surviving cells of certain tissues are necessary for the growth of vaccinia virus *in vitro*; the essential conditions which they provide are at present unknown, and cannot otherwise be provided at will. We have not yet been able to repeat the results of Eagles and McClean. Until the growth requirements of the virus are more fully understood, or until the conditions for cultivation of the virus in the absence of surviving cells can be defined and obtained consistently, the significance of an occasional apparently successful series of cultures in a cell-free medium will remain obscure.

It is relevant that Rivers, Haagen and Muckenfuss (1929) found that repeated freezing and thawing of hen's kidney destroyed its growth-promoting property. Muckenfuss and Rivers (1930) and Muckenfuss (1931) obtained no increase of virus with dead (frozen and thawed) cells when this system was separated by a collodion membrane from surviving cells.

SUMMARY.

1. The observation of others that vaccinia virus will multiply in a medium consisting of suitable minced tissue in Tyrode's solution, without the addition of serum, has been confirmed.

2. The virus grown in minced rabbit kidney and Tyrode's solution maintained its infectivity well when stored at $\pm 5°$ C.

3. A proper ionic composition of the saline constituents of the medium is essential for growth of the virus. Tyrode's solution was suitable, but buffered phosphate solution was not. It is suggested that a probable explanation is that the tissue cells failed to survive in an unbalanced serum-phosphate mixture.

4. Ready access of oxygen to the tissue in the medium is essential for growth of the virus. The virus failed to multiply when the tissue was covered by a layer of liquid 15 mm. deep, and under these conditions respiration of the tissue rapidly ceased.

5. The period for which minced rabbit kidney could be incubated at 37° C. prior to inoculation, and still serve for cultivation of the virus, corresponded roughly with the period during which it respired.

6. A preliminary survey of the relation between the respiratory activity of cells surviving in a medium and the conditions required for the growth of the virus indicated (*a*) a correlation between the respiratory activity of different tissues and their suitability to promote growth of virus; (*b*) a correlation between the period for which a suitable tissue retained, during incubation in a saline medium at 37° C. prior to inoculation, its property of promoting multiplication of virus and the period for which it respired; and (*c*) a correlation between the unsuitability of kidney in test-tubes under a layer of medium 15 mm. deep and the absence of respiration.

7. We have, so far, been unable to obtain cultures in a cell-free medium (kidney extract). Virus that had survived for 2 to 4 days in such a medium failed to grow when subcultured to a similar medium, but multiplied when transferred to a medium containing minced kidney.

8. The experiments of this paper suggest that the conditions provided by suitable surviving cells for cultivation of the virus cannot at present otherwise be obtained at will. In the absence of fuller knowledge of the growth requirements of the virus the significance of occasionally successful cultures in a cell-free medium remains obscure.

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THE CURATIVE ACTION OF ANTI-PNEUMOCOCCUS SERUM (TYPE I) IN MICE.

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THE titration of the protective antibody in anti-pneumococcus sera (Type I) is still in the experimental stage. A method which is used by some workers consists of the simultaneous injection into the peritoneal cavity of mice of a test-dose of the culture, together with graduated doses of the serum.

In the course of experimental work on the titration of anti-pneumococcus sera we sought to ascertain how the infection progressed in mice when a small

dose of Type I pneumococci was given to them, and how the specific serum influenced the infective process at different stages of its development. The experiments we have carried out indicate that the serum is able to control the infection even when a bacteriæmia is present, and thus the inference may be drawn that it possesses a curative action.

THE PLAN AND THE TECHNIQUE OF THE EXPERIMENTS.

The experiments were arranged according to the following scheme :—A small and constant dose of an 18-hour broth culture of a virulent strain of Type I pneumococcus was injected intraperitoneally into a number of mice, and thereafter at regular intervals small groups of the mice were killed and examined. Some of the mice served as controls, and the others received a single dose of a specific serum intravenously at varying intervals after the dose of culture By this means a comparison could be made between the control mice and the serum-treated mice at any stage of the infective process. In some of the experiments the fatality-rate in a group of serum-treated mice was compared with the rate in a group of control mice.

Each group of mice was chloroformed to death at the pre-arranged time, and peritoneal smears and cultures were taken. When fluid was present during the early hours of the infection—that is, up to 4 hours—the peritoneal culture was made from a single drop of the fluid, which was run over an agar slope ; the fluid probably consisted for the most part of the broth in which the dose of cocci was suspended. At later stages a wire loop was drawn lightly across the peritoneal surface of the liver or the intestines ; usually it was not possible to carry over a film of fluid to the culture-tubes.

A culture was made from the heart by introducing two drops of the blood from a fine capillary pipette into one or two agar-slope tubes, and by tilting the blood first into the condensation fluid and afterwards over the slope. The tubes were subsequently maintained upright so that the colonies might develop on the surface of the medium. The amount of blood from each mouse was estimated to be 0·1 c.c., but since most of it drained back into the condensation fluid, the colonies which developed originated from only a fraction of this amount. An elaborate technique was avoided, in order that the times indicated in the scheme of the experiment might be adhered to as closely as possible. When the colonies on an agar-slope were very numerous and discrete, an estimate of them in round numbers was made. We are satisfied that the figures which indicate the degree of the bacteriæmia and of the peritoneal infection, although only approximate, are sufficiently comparable for the purpose we had in view.

Heart-blood smears were examined in one experiment, but were thought not to be worth repeating, for diplococci are seen only when a heavy bacteriæmia is present. On some occasions smears of the organs were also examined ; Giemsa's stain and Hiss's capsule stain were used.

The Lethal Power of the Strain of Type I Pneumococcus used in the Experiments.

Table I gives some details of the lethal power of the strain we have used during the past two years ; a full account will be found in a recent publication

(Petrie and Morgan, 1931). In the experiments we are now describing a constant dose of $1/(10^7)$ c.c. of a young broth culture was chosen ; we estimate this dose to contain about 50 diplococci ; the volume of the inoculum was 1 c.c. The mortality among mice receiving the dose is a high one—93 per cent. —and the mean death-time is 45 hours—a figure which clearly has an important relation to the time at which the serum was given.

TABLE I.—*Giving Details of the Lethal Power of the Strain of Type I Pneumococcus Used in the Experiments.*

Dose (c.c.).	Number of mice injected.	Percentage of deaths.	Mean death-time (hours).
$1/(10^7)$	164	93	45
$1/(10^8)$	769	75	51

TABLE II.—*Showing the Progress of the Infection in Mice at 2-Hourly Intervals after an Intraperitoneal Dose of $1/(10^7)$ c.c. (about 50 Diplococci) of a Virulent Culture of Type I Pneumococcus had been given.*

Group (3 mice in each).	Interval between dose of culture and examination (hours).	Peritoneal smear (cocci).	Peritoneal culture (colonies).		Heart-blood culture (colonies).	
			Individual counts.	Total count.	Individual counts.	Total count.
A	2	0	0, 0, 0,	0	0, 0, 0	0
B	4	0	2, 0, 4	6	0, 0, 3	3
C	6	0	5, 21, 10	36	5, 0, 2	7
D	8	0	7, 61, 17	85	83, 5, 8	96

The Progress of the Infection in the Mice.

A consideration of the data which have been arranged in Table II indicates the absence of any considerable lag in the multiplication of the cocci in the peritoneal cavity. This observation is in agreement with evidence we have previously brought forward (1931) that a minimal dose of our strain of pneumococcus, even when only one diplococcus is injected, is capable of establishing itself in the peritoneal tissues with almost the same degree of certainty as in a serum-broth tube. When 6 hours have elapsed after the dose of culture is given, a cellular exudate is apparent on microscopical examination ; this consists of a variety of cells, which include polymorphonuclear cells, mononuclear cells, large phagocytic cells with vacuolated protoplasm and mast-cells.

The onset of the bacteriæmia is also rapid, for colonies were obtained from the heart-blood in from 2 to 4 hours after the infecting dose was given to the mice.

The experiment recorded in Table IV carries the observations to a later stage, namely, up to 18 hours after the culture dose was given. The data show that the infection in the primary focus—the peritoneum—is proceeding with increasing intensity, presumably until death, and that the infection in the blood keeps pace with it.

THE THERAPEUTIC EFFECT OF A SINGLE DOSE OF A SPECIFIC SERUM WHEN GIVEN INTRAVENOUSLY AT VARYING INTERVALS AFTER THE INFECTING DOSE OF CULTURE.

We used in these experiments three samples of serum: "A," a natural serum with about 100 Felton units* per c.c., "B," a natural serum with about 700 Felton units per c.c., and "C," a concentrated preparation with about 2500 Felton units per c.c. These estimates are given merely for the sake of comparison, and with the knowledge that they are only approximate, because a recognized dry standard serum of definite unitage is not available at the present time.

TABLE III.—*Showing the Influence upon the Survival-rate in Mice of a Single Dose of Serum "A," when Administered Intravenously at Varying Times after an Intraperitoneal Dose of* $1/(10^7)$ *c.c. (about 50 Diplococci) of a Virulent Pneumococcus Culture of Type I had been given.*

Date.	Number of tests.	Dose of serum.	Interval between dose of culture and dose of serum (hours).	Control mice. Deaths/Total.	Serum mice. Deaths/Total.
1.iv.31 to 12.vi.31	5	0·5 c.c.–0·004 c.c. (50 "units" –0·33 "unit")	7	26/30 (86·7%)	5/85 (5·9%)
17.iv.31	1	0·5 c.c. (50 "units")	15	9/10	8/10
1.iv.31	1	Ditto	19	10/10	10/10

Table III shows the influence upon the survival-rate in mice of a single dose of serum "A" when it was administered intravenously 7 or 15 or 19 hours after an intraperitoneal dose of about 50 diplococci. Doses of from 50 "units" to 0·33 "unit," when given 7 hours after the dose of culture, reduced the mortality from 87 per cent. in the control mice to 6 per cent. in the serum-treated animals. Fifty "units" of this serum failed to protect the mice when given 15 or 19 hours after the infecting dose. A culture was taken from the heart-blood of 19 mice which survived for 4 days, the time-limit of the test; it proved to be sterile in every instance.

Table IV gives the details of a similar experiment in which a single dose of 50 "units" of serum "A" was given intravenously 7 or 12 or 16 hours after the infecting dose. Observations were made on the course of the infection by sacrificing small groups of mice at successive intervals of 2 hours and examining peritoneal smears and heart-blood cultures. The results make it evident that, when the serum was given 7 hours after the infecting dose, a vigorous clearing process reduced the infection in the peritoneum and the blood-stream when the serum had acted for only 1 hour. The same dose of serum was given 12 hours after the infecting dose, and although at the time of its administration the infection was a severe one—the colonies from the heart-blood of the corresponding control mice were uncountable—nevertheless a rapid reduction in the bacteriæmia was evident at the end of 2 hours, and when

* A Felton unit is the amount of specific serum which, when injected intraperitoneally into mice together with a test-dose of a young, highly virulent culture, is capable of neutralizing one million minimal lethal doses of the culture.

TABLE IV.—*Showing the Course of the Infection in Mice at 2-Hourly Intervals after an Intraperitoneal Dose of* $1/(10^7)$ *c.c. (about 50 Diplococci) of a Virulent Culture of Type I Pneumococcus:* (1) *In Control Groups, and* (2) *in Groups which Received a Single Intravenous Dose of a Specific Serum (Serum "A") 7, 12 and 16 Hours after the Injection of the Infecting Dose.*

Control groups (3 mice in each).			Serum groups (2 mice in each). 0·5 c.c. (50 Felton units) of specific serum given intravenously at 7 hours.			Serum groups (2 mice in each). 0·5 c.c. (50 Felton units) of specific serum given intravenously at 12 hours.			Serum groups (2 mice in each). 0·5 c.c. (50 Felton units) of specific serum given intravenously at 16 hours.		
Interval between dose of culture and examination (hours).	Pneumococci in peritoneal smear.	Heart-blood culture (colonies).	Time of action of serum (hours).	Pneumococci in peritoneal smear.	Heart-blood culture (colonies).	Time of action of serum (hours).	Pneumococci in peritoneal smear.	Heart-blood culture (colonies).	Time of action of serum (hours).	Pneumococci in peritoneal smear.	Heart-blood culture (colonies).
2	0	0, 1, 1	—	—	—	—	—	—	—	—	—
4	±	3, 12, 10	—	—	—	—	—	—	—	—	—
6	±	8, 5, 1	—	—	—	—	—	—	—	—	—
8	?±	300, 20, 150	1	±	0, 0	—	—	—	—	—	—
10	+	1000, 1000, 200	3	±	0, 0	—	—	—	—	—	—
12	++	2000, 2000, 2000	5	±	0, 0	—	—	—	—	—	—
14	++	∞, ∞, ∞	7	±	0, 0	2	+	14, 1	—	—	—
16	+++	∞, ∞, ∞	9	±	0, 0	4	++	100, 0	—	—	—
18	+++	1000, 500, 2000	11	0	0, 0	6	±	0, 0	2	+++	∞, ∞

Note.—± = scanty; + = fair numbers; ++ = numerous; +++ = very numerous; ++++ = like a culture smear.

TABLE V.—*Showing the Course of the Infection in Mice at 3-Hourly Intervals after an Intraperitoneal Dose of* $1/(10^7)$ *c.c. (about 50 Diplococci) of a Virulent Culture of Type I Pneumococcus: (1) In Control Groups, and (2) in Groups which Received a Single Intravenous Dose of a Specific Serum (Serum "A") 12 and 15 Hours after the Injection of the Infecting Dose.*

	Control groups. (2 mice in each).			Serum groups (2 mice in each). 0·5 c.c. (50 Felton units) of specific serum given intravenously at 12 hours).			Serum groups (2 mice in each). 0·5 c.c. (50 Felton units) of specific serum given intravenously at 15 hours.		
Interval between dose of culture and examination (hours).	Pneumococci in peritoneal smear.	Heart-blood culture (colonies).		Time of action of serum (hours).	Pneumococci in peritoneal smear.	Heart-blood culture (colonies).	Time of action of serum (hours).	Pneumococci in peritoneal smear.	Heart-blood culture (colonies).
12	±	50 / 12	.	—	—	—	—	—	—
15	++	100 / 100	.	3	0	0 / 0	—	—	—
18	++	50 / 500	.	6	0	0 / 0	3	+	0 / 0
21	+++	1000 / 500	.	9	0	0 / 0	6	++	0 / 0
24	++++	2000 / 500	.	12	0	0 / 0	9	+	0 / 0
36	++++	∞ / ∞	.	24	0	0 / 0	21	0	0 / 0

Note.—± = scanty; + = fair numbers; ++ = numerous; +++ = very numerous; ++++ = like a culture smear.

the serum had acted for 6 hours no colonies appeared in the cultures. The same dose of serum was given intravenously to 2 mice 16 hours after the infecting dose ; these were killed 2 hours later, and both showed a heavy infection of the peritoneum and the heart-blood—a result which indicates that the serum had been administered too late to be effective. Thus, in this experiment the serum proved to be capable of controlling the infection as late as 12 hours after the infecting dose—an interval of time which represents one-quarter of the whole infective period, on the average, from the time of infection of the mice until their death (see Table I).

The experiment of which the details are given in Table V was carried out on similar lines and gave similar results. The mice received a single intravenous dose of 50 " units " of serum " A " 12 or 15 hours after the infecting dose. The curative action of the serum on the primary focus of the infection—the peritoneum—and on the blood is evident.

Table VI illustrates the curative action of serum " B " at a stage of the infective process when a bacteriæmia is present.

TABLE VI.—*Illustrating the Curative Action of Anti-pneumococcus Serum (Type I) in Mice at a Stage of the Infective Process when a Bacteriæmia was Present. All of the 60 Mice Referred to in this Table received, at about the same time, an Intraperitoneal Dose of* $1/(10^7)$ *c.c. (about 50 Diplococci) of a Virulent Culture of Type I Pneumococcus. Thirty Mice served as Controls, and 30 received an Intravenous Dose of Serum "B" 12 Hours after the Injection of the Infecting Dose.*

Control group. Killed at 12 hours.		Control group. Killed at 15 hours.		Serum group. Serum given intravenously at 12 hours. Killed at 15 hours.	
Number in group.	Heart-blood culture (colonies).	Number in group.	Heart-blood culture (colonies).	Number in group.	Heart-blood culture (colonies).
10	10–1000 (7>100)	10	65–∞	10	8 cases = 0 1 case = 2 1 ,, = 4

Control group.		Serum group. Serum given intravenously at 12 hours.	
Number in group.	Deaths.	Number in group.	Deaths.
10	9/10	20	0/20

In this experiment 60 mice received at about the same time an intraperitoneal dose of about 50 diplococci. Thirty mice served as controls, and 30 received an intravenous dose of 0·5 c.c. of serum " B " (350 " units ") 12 hours after the inoculation of the infecting dose, namely, after one-quarter of the total infective period had passed. First, a control group of 10 mice were killed when 12 hours had elapsed, and cultures were made from the heart-blood. The number of colonies varied from 10 to 1000, and there were more than 100 colonies from the heart-blood of 7 of the mice. Three hours later a second control group of 10 mice were killed. The colonies from the heart-blood were naturally more numerous than in the first group ; they varied from 65 to an uncountable number. At an interval of 12 hours after the infecting dose—that is, at a time when the bacteriæmia was of a degree which corresponded to that in the first control group—10 mice were given 0·5 c.c. of serum " B," and were

killed and examined 3 hours later—that is, at a time when the bacteriæmia was of a degree which corresponded to that in the second control group. No colonies grew in the culture-tubes prepared from the heart-blood of 8 of the mice ; in one instance there were two colonies, and in one there were four colonies.

From these observations alone it cannot be stated with certainty that the curative action was not merely a temporary one, and that death might not have followed. As a further control, therefore, the infection in 10 mice was allowed to run its course, with the result that 9 out of the 10 died, thus demonstrating that the lethal power of the culture used in this experiment was normal (see Table I). Again, a group of 20 mice, each of which received 0·5 c.c. of serum " B " intravenously 12 hours after the infecting dose, were allowed to run their course ; they remained quite healthy, and all were alive 12 days later.

THE TITRATION OF THE CURATIVE DOSE OF A SPECIFIC SERUM.

In the experiment which has just been described, the dose (0·5 c.c.) of serum " B " that was administered intravenously represents about 350 Felton units—an amount of antibody which is in excess of the dose required to protect 50 per cent. of the mice from death under the conditions of the experiment. A test was therefore carried out to determine the dose which would give an even chance of life or death among a group of mice to which it was administered. The results are set forth in Table VII, and they show that, as the dose of serum is progressively diminished, the fatality-rate in the mice increases, so that 1/750 c.c. of the serum confers no protection. The groups are too small for accurate titration, but the results indicate that the curative dose of serum

TABLE VII.—*Showing the Results of Titrating by the Intravenous Route the Curative Dose of Serum " B " which was Used in the Experiments set forth in Table VI. The Results also Demonstrate the Relation between the Time at which the Serum was Given and its Curative Action.*

Dose of serum (c.c.).	Serum given 12 hours after an infecting dose of about 50 diplococci.		Serum given 15 hours after an infecting dose of about 50 diplococci.	
	Deaths/mice inoculated.	Percentage deaths.	Deaths/mice inoculated.	Percentage deaths.
1/10	0/10	..	8/10	80
1/25	..	..	6/10	60
1/50	2/10	20	9/10 (M.D.T.* 47 hrs.)	90
1/100	6/19	32	9/10 (M.D.T.* 36 hrs.)	90
1/175	14/20	70	..	..
1/250	11/20	55	..	..
1/350	15/20	75	..	..
1/500	9/10	90	..	..
1/750	10/10	100	..	..
Controls	17/20	85	..	..

* Mean death-time.

" B," when given 12 hours after the infecting dose of culture, is about 1/200 c.c. (3·5 " units "); this interpretation of the data assumes that the fatality-rate among the mice which received 1/175 c.c. is unduly high owing to sampling errors.

The curative action of Serum " C," a concentrated product which was estimated to contain 2500 Felton units per c.c., was tested in precisely the same way as the natural sera "A" and " B." This preparation was given to the mice intravenously 12 hours after the infecting dose, and it was found that the curative dose was 1/400 c.c.—an amount which contains approximately 6 " units " of antibody.

The data in Table VII further show clearly the relation between the time of administration of the serum and its curative action. When the doses of serum were given 15 hours after the infecting dose of culture, the conditions being otherwise equal, even 1/10 c.c. failed to protect 50 per cent. of the mice. Direct observation of the degree of infection at the times when the doses of serum were administered was made by killing and examining 5 control mice 12 hours after the infecting dose ; cultures from the peritoneum yielded from 500 to 5000 colonies, and the colonies from the heart-blood varied from 170 to 400 approximately. Again, 5 control mice were killed and examined 15 hours after the infecting dose ; colonies which were too numerous to count grew from the peritoneum, and from 40 to 2000 colonies grew from the heart-blood.

THE INFLUENCE OF THE TIME OF ACTION OF THE SERUM UPON AN ESTABLISHED INFECTION.

The results shown in Tables IV, V and VI indicate that the serum acts energetically and rapidly on the infection in both the peritoneal tissues and the blood, because a marked reduction of the infection was manifest after it had acted for as short a period as 1 hour. An experiment was planned to ascertain whether the serum was able to exert its effect within an even shorter period ; the results are assembled in Table VIII. A dose of 1/100 c.c. of serum " B," an amount which contains about 7 Felton units, was given intravenously to 15 mice that had received the standard dose of culture 12 hours previously. The mice were then killed in groups of three at intervals of 5, 15, 30, 60 and 120 minutes ; these figures represent the time of action of the serum on the infective process. Smears of the peritoneum and the spleen were prepared from all the mice, and peritoneal and heart-blood cultures were made from one mouse of each group. The whole of the observations were duplicated in a series of mice which received 0·5 c.c. of normal horse-serum ; as was expected, these mice showed no evidence of having been protected, and they may, therefore, be regarded as controls to the observations which were made on the serum-treated groups. As further controls, 5 mice which received the standard dose of culture were observed for the usual test period, and during this time all of them died, with a mean death-time of 39 hours ; 5 mice which received 0·5 c.c. of normal horse-serum intravenously died, with a mean death-time of 36 hours ; whereas of 5 mice which received 1/100 c.c. of Serum " B " intravenously, only 1 died.

The results of this experiment, which are summarized in Table VIII, again demonstrate the powerful action of the specific serum on the bacteriæmia.

Moreover, they indicate that the serum produces its effect with great rapidity, since the blood infection appears to show a reduction even after the serum had acted for only 5 minutes. The examination of the peritoneal smears gave less evidence of rapid action, but there was a definite reduction in the infection, as compared with the control mice, after the serum had acted for 1 hour and for 2 hours.

TABLE VIII.—*Showing the Influence upon the Bacteriæmia and the Peritoneal Infection in Mice of a Single Intravenous Dose of a Specific Serum when given* 12–14 *Hours after an Intraperitoneal Dose of* 1/(10^7) *c.c.* (*about* 50 *Diplococci*) *of a Virulent Culture of Type I Pneumococcus. The Time of Action of the Specific Serum* (*Serum "B"*) *varied from* 5 *Minutes to* 2 *Hours, and a Normal Serum Control was used.*

Interval between dose of culture and examination.	Time of action of serum (minutes).	0·5 c.c. normal horse-serum intravenously.		1/100 c.c. specific serum intravenously.	
		Heart-blood culture (colonies).	Peritoneal culture (colonies).	Heart-blood culture (colonies).	Peritoneal culture (colonies).
12 hrs. 5 min.	5	63	∞	3	80
12 ,, 15 ,,	15	100	∞ *	5	∞
12 ,, 30 ,,	30	1 (?)	∞ *	1	∞
13 ,,	60	∞	∞	1	∞
14 ,,	120	∞	∞	5	∞

THE MODE OF ACTION OF THE SERUM AS A CURATIVE AGENT IN MICE.

At the present time the mode of action of anti-pneumococcus serum is not fully understood, and there is no general agreement as to the relative importance of such mechanisms as phagocytosis and agglutination. Neufeld and Schnitzer (1928), for example, believe that phagocytosis is the only protective mechanism for which there is adequate proof, although they admit that some unknown factors may play a part. In a recent publication Avery and Dubos (1931) have brought forward confirmatory evidence of a remarkable character, which shows that the phagocytic process is intimately concerned with the presence or absence of the polysaccharide-containing capsule of the pneumococcus. They have proved that pneumococci (Type III) which have suffered loss of the capsular polysaccharide by enzymic action are readily phagocyted. Bull (1915) thinks that agglutination of the pneumococci is an important part of the immunity reaction, and that the phagocytes can then deal with the clumps of cocci; he used rabbits in his experiments and gave both the cocci and the serum intravenously. The suggestion has been made that the serum has an antitoxic action, but it is generally conceded that there is scanty experimental evidence for the existence of a specific pneumococcal toxin. In the opinion of Griffith (1929), however, the facts seem to indicate that in acute lobar pneumonia in man the pneumococcus secretes a diffusible toxin.

Our work does not permit a definite conclusion to be drawn from it upon the mode of action of the serum, but we formed the impression that the amount of phagocytosis was less than the efficacy of the serum treatment might indicate. The peritoneal smears from the *control* mice in the experiment recorded in

* The colonies in these cultures were two or three times as dense as those in the corresponding peritoneal culture of the mouse which received the specific serum.

Table IV showed little or no phagocytosis up to 16 hours. Polymorphonuclear cells could be seen at this time with a half ring of cocci or even with one or two complete rings of cocci encircling them, but with no cocci within the cell. However, in the next group of control mice, which were killed after an interval of 18 hours, good phagocytosis was seen in the peritoneal smear. In the smear from one mouse in this group the phagocytes were chiefly large cells with vacuolated protoplasm ; the polymorphonuclear cells were mostly empty. In the smear from another mouse in this group polymorphonuclear cells predominated, and there was good phagocytosis, although the " ring " phenomenon was also noted. Phagocytosis may signify an attempt at a defence reaction at this stage of the infective process. When the control series of mice in this experiment is compared with the serum-treated mice in respect of phagocytosis, there does not seem to be any marked difference between them. In one of the mice that was given serum 16 hours after the infecting dose, it was noted that pneumococci were present in large phagocytic cells, and that there were clear haloes round some of these diplococci. Numerous pneumococci were present in the peritoneal smear which was taken from the companion of this mouse ; some of the cocci formed half rings or rings round the cells or cell-groups.

These observations led us in the next experiment (see Table V) to seek for evidence of phagocytosis in impression smears of the spleen, the lung, the liver and the bone-marrow of the mice. The peritoneal smears in both the control and the serum-treated mice resembled those which have just been described. In the control mice the spleen showed microscopical evidence of invasion after an interval of from 15 to 18 hours, and it contained numerous diplococci after an interval of 24 hours. Up to 24 hours pneumococci were not constantly seen in the smears of the lung, the liver and the bone-marrow ; the last-mentioned tissue showed the fewest cocci. The serum-treated mice differed from the control mice in the complete absence, on microscopical examination, of free cocci in the smears from the spleen, the lung, the liver and the bone-marrow ; and yet there was scanty evidence of phagocytosis.

The smears of the peritoneum and the spleen which were taken from the mice in the experiment recorded in Table VIII confirmed the previous findings, for phagocytosis was not an obvious feature. One of the spleen smears from a mouse in which the specific serum had been acting for 2 hours showed exceptionally good phagocytosis ; the cells chiefly concerned were large mononuclear phagocytes.

This experiment was repeated with the following modifications : (1) The specific serum was given intraperitoneally instead of intravenously ; (2) the dose of serum (1/100 c.c. serum " B ") was given 14 hours after the infecting dose instead of 12 hours, in order to ensure a considerable degree of infection and thus facilitate observation of the phagocytic process, and (3) the normal serum control was omitted.

After the serum had acted for 1 hour the clearing action on the infection in the peritoneum and the blood, as judged by the cultural findings, was quite definite. Phagocytosis was readily observed in the peritoneal smears which were taken after the serum had acted for 5 minutes and for 15 minutes, but it was an inconspicuous feature in the peritoneal smears which were taken after the serum had acted for 30, 60 and 120 minutes.

When the serum was given intravenously, there was no evidence of agglutination of the cocci in any of the preparations ; when it was given intraperitoneally there was very little evidence of agglutination of the free cocci in the peritoneal tissues, in spite of the direct action of the serum on the cocci.

DISCUSSION.

A brief reference may be made to work that has already been published on curative experiments in animals. Wadsworth (1912) administered to 8 rabbits a single dose (2 c.c.) of a specific serum, ½ hour, 1 hour, 3 hours and 7 hours after an intravenous injection of 0·25 c.c. of a virulent culture (? type). The animals that received the serum ½ hour and 1 hour after the infecting dose recovered. Bull (1915) infected rabbits intravenously with a virulent culture of Type I pneumococcus, and, after 2 minutes had elapsed, treated them with repeated small doses of serum given intravenously until the 56th hour ; 4 rabbits treated in this way recovered. In 1920 Cecil and Blake published their experiments on the curative action of anti-pneumococcus serum (Type I) in 5 monkeys (*M. syrichtus*) ; a pneumonia was produced in these animals by the intratracheal injection of the virulent culture, and from 60 to 150 c.c. of a specific serum was then given to them intravenously in 10 c.c. or 20 c.c. doses. The serum was effective when a bacteriæmia was already present, and when the treatment was begun as late as 4 days after the infecting dose. Cecil and Steffen (1925) produced an experimental Type I pneumonia in 2 monkeys, and successfully treated them with Huntoon's multivalent antibody solution 3 days after the infecting dose had been given. Coventry (1927) saved 7 out of 13 mice by administering to them a concentrated serum preparation 4 hours after the injection of 1000 lethal doses of a virulent Type I culture ; both culture and serum were given intraperitoneally. Finally, Goodner (1928) found that the intravenous administration to rabbits of 4 c.c. of a specific serum brought about recovery at a time when the blood yielded 50 colonies of pneumococcus per c.c. ; the serum was given 24 hours after an intracutaneous dose of virulent Type I pneumococci.

The results we have obtained make it clear that an anti-pneumococcus serum (Type I) is capable of exerting a curative action in mice even when a bacteriæmia is present. The fate of the mice is apparently determined by three factors, namely, the amount of antibody injected, the time of its injection in relation to the infecting dose, and the degree of infection at the time of its administration. There is naturally a limit of time beyond which the administration of the serum cannot prevent death.

The results appear to possess a three-fold significance. In the first place they strengthen the idea that the mouse titration method is capable of measuring the potency of the serum as a remedial agent for the treatment of pneumonia in man. Secondly, they support the belief that the specific antibody in anti-pneumococcus serum is really capable of combating and reducing a bacteriæmia in man ; the prognosis in lobar pneumonia is known to be much worse when a bacteriæmia is present. And lastly, the results indicate that a curative test on a similar plan to those we have carried out may prove to be satisfactory as a method of titrating the curative antibody in anti-pneumococcus sera. We hope to examine this possibility.

CONCLUSIONS.

1. A single dose of a natural anti-pneumococcus serum (Type I), when given intravenously to mice up to 12 hours after an infecting dose of a highly virulent culture, is able to cure the infection, even when a bacteriæmia is present. The infecting dose employed in these experiments contains about 50 diplococci, and is fatal to 93 per cent. of control mice. The interval of 12 hours between the infecting dose and the dose of serum represents one-quarter of the mean death-time of control mice which receive this dose of culture.

2. A concentrated preparation of the specific antibody (Type I) is also able to exert a curative action under similar conditions.

3. The relation between the time of administration of the serum and its curative effect is clearly demonstrable from the experiments. A dose of serum which is adequate to exert a curative effect when it is administered 12 hours after the infecting dose, must be increased about 100 times in order to be effective when the serum is given 3 hours later.

4. The serum, when it is given intravenously, acts with great rapidity on the bacteriæmia. The efficacy of the intravenous method of treatment and the increased risk of death from delaying serum treatment for even a few hours emphasize the importance, in the treatment of acute lobar pneumonia in man, of administering the specific serum by the intravenous route at the earliest possible moment.

5. The curative dose of the serum at a particular stage of the infective process can be determined by administering graduated doses to groups of mice, and by noting the dose which gives a 50 per cent. survival-rate. Tests of this kind may prove to be applicable as a method of titrating the curative antibody in anti-pneumococcus serum.

6. There is no reason to doubt the importance of the phagocytic mechanism in the clearing process, which quickly follows the administration of the serum to mice, and yet in our experience it is less apparent than the efficacy of the serum treatment indicates. When the serum is given intravenously, phagocytosis is less easily observed than when it is administered directly into the primary focus of the infection, namely, the peritoneal tissues, but even here phagocytosis can scarcely be said to be a conspicuous feature of the microscopical picture.

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